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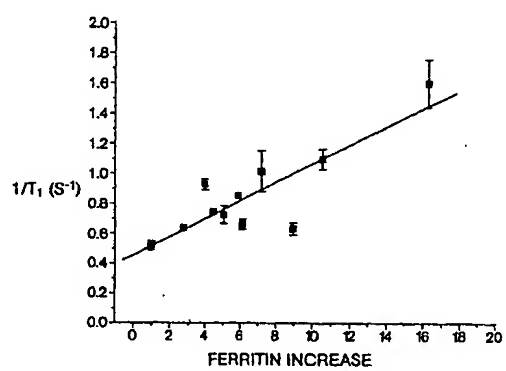
(54) Title: CONTRAST AGENTS FOR MAGNETIC RESONANCE IMAGING AND METHODS RELATED THERETO

(57) Abstract: In certain aspects of the present invention provides methods and compositions related to contrast agents for magnetic resonance imaging. In certain variations, contrast agents provided herein are generated in situ via genetic instructions and become potent upon sequestering available metal atoms. Exemplary contrast agents include metal-binding proteins.



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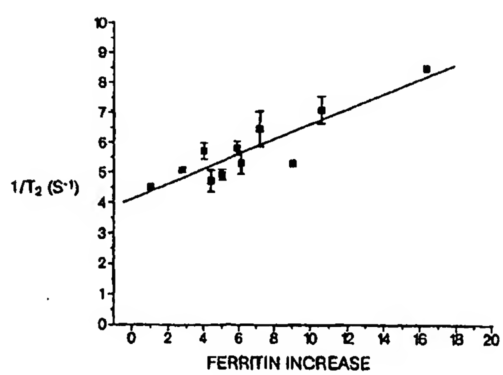

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CONTRAST AGENTS FOR MAGNETIC RESONANCE IMAGING AND METHODS RELATED THERETO

INTRODUCTION

Tools that enable one to visualize gene expression in vivo are of fundamental importance to the future of medicine and the biological sciences. The emerging field of genetic medicine requires non-invasive imaging methods that can indicate where, when and if therapeutic genes have been delivered and whether the desired protein has been expressed. In the realm of basic biological research, the ability to image the timing and location of gene expression in vivo is a fundamental need.

Scientists typically monitor gene expression by incorporating a marker gene that is expressed along with the gene of interest, often as either a transcriptional or translational fusion. Detection of the marker gene products is most often achieved using histological preparations (e.g. using a β-galactosidase assay), or by using fluorescence microscopy (e.g. using green fluorescent protein, or GFP). Neither of these methods permit non-invasive imaging of tissues or other macroscopic assemblies of cells. Markers that require histological preparation cannot be detected without sacrificing the subject material. Fluorescent markers can be imaged in living cells, but even with the most sophisticated optical technologies available, it is not possible to image at tissue depths exceeding approximately 500 μm. Other methods such as PET (positron emission tomography), gamma cameras, and SPECT (single-photon emission computed tomography) have been used to detect gene expression in vivo, but all of these suffer from limited spatial resolution, which is on the order of cubic millimeters or larger.

MRI is a widely used clinical diagnostic tool that allows non-invasive imaging of optically opaque subjects and provides contrast among soft tissues at high spatial resolution. In the majority of clinical applications, the MRI signal is derived from protons of the water molecules present in the materials being imaged. The image intensity of

tissues is determined by a number of factors. The physical properties of a specific tissue, such as the proton density, spin lattice relaxation time (T1), and the spin-spin relaxation time (T2) often determine the amount of signal available.

A number of compositions termed "contrast agents" have been developed to provide enhanced contrast between different tissues. Contrast agents commonly affect T1, T2 or both. In general, contrast agents are made potent by incorporating metals with unpaired *d* or *f* electrons. For example, T1 contrast agents often include a lanthanide metal ion, usually Gd^{3+}, that is chelated to a low molecular-weight molecule in order to limit toxicity. T2-agents often consist of small particles of magnetite ($FeO\text{-}Fe_2O_3$) that are coated with dextran. Both types of agents interact with mobile water in tissue to produce contrast; the details of this microscopic interaction differ depending on the agent type.

Most widely used contrast agents are exogenous, meaning that the contrast agent is produced externally and then delivered to the tissue or cells to be imaged. Exogenous contrast agents are generally delivered through the vascular system, typically have a nonselective distribution, and are physiologically inert. The exogenous contrast agents are used to highlight anatomy with poor intrinsic contrast, as well as to visualize various pathologies that disrupt normal vascular flow or cause a break in the blood-brain-barrier. None of these agents cross cellular membranes easily and therefore the existing technology is difficult to adapt for the analysis of intracellular events.

A new generation of MRI contrast agents is required to adapt this powerful imaging technology to the needs of molecular medicine and biological research.

SUMMARY OF THE INVENTION

In certain aspects, the invention relates to contrast agents for magnetic resonance imaging that are synthesized in a subject material as directed by a nucleic acid sequence.

The contrast agents are made potent by sequestering available metal atoms, typically iron atoms. In certain aspects, the nucleic acid sequence encodes a metal binding protein that acts, directly or indirectly, to impart a contrast effect on the cell in which it is produced. The invention further relates to methods of generating and employing the subject contrast agents.

In certain embodiments, the invention relates to methods of generating an image of a subject material by imaging a subject material comprising a plurality of cells wherein a subset of the cells contain an MRI-detectable amount of contrast protein. In preferred embodiments, the amount of contrast protein present in different cells is distinguishable, and optionally, cells comprising measurable amounts of contrast protein are distinguishable from cells or other components of the material that do not comprise the measurable amount of contrast protein.

In another embodiment, methods of the invention comprise detecting gene expression by imaging a cell comprising a recombinant nucleic acid encoding a contrast agent. Preferably, detection of the contrast protein by magnetic resonance imaging indicates that the nucleic acid encoding the contrast protein is and/or has been expressed. Optionally, the contrast agent is a protein, preferably a metal-binding protein. Exemplary classes of metal binding proteins include ferritin proteins; transferrin receptor proteins; iron regulatory proteins; and iron scavenger proteins. Exemplary metal binding proteins of the invention include metal binding proteins that are at least 60%, optionally at least 70%, 80%, 90%, 95%, 99% or 100% identical to a sequence as shown in any of SEQ ID Nos: 2, 4, 6, 8, 10, 12, and 14. Alternatively, the protein is at least 60%, optionally at least 70%, 80%, 90%, 95%, 99% or 100% identical to a sequence as shown in any of SEQ ID Nos: 16, 18, 20 or 22.

Methods described herein may be used with essentially any material capable of generating the contrast agent in situ. For example, the subject material may be a cell, optionally a cell that is part of a cell culture, part of an in vitro tissue or part of a multicellular organism, such as, for example, a fungus, a plant, or an animal. In preferred embodiments, the subject material is a living mammal such as a mouse or a human.

In further aspects, the invention provides vectors for transfection of a multicellular organism comprising a recombinant nucleic acid encoding a contrast agent. In certain embodiments, the contrast agent is a metal-binding protein. Optionally, the vector is a viral vector derived from a virus selected from the group: an adenovirus, an adenovirus-associated virus, a herpes simplex virus, a retrovirus, an alphavirus, a poxvirus, an arena virus, a vaccinia virus, an influenza virus, a polio virus and a hybrid of any of the foregoing.

In additional aspects, the invention includes delivery systems for introducing nucleic acids of the invention into subject material. In certain embodiments, the invention provides viral particles suitable for transfecting a mammalian cell, comprising a nucleic acid comprising a coding sequence for a contrast agent, such as a contrast agent described above. Optionally, the viral particle is derived from one or more of the following: an adenovirus, an adenovirus-associated virus, a herpes simplex virus, a retrovirus, an alphavirus, a poxvirus, an arena virus, a vaccinia virus, an influenza virus and a polio virus. In additional embodiments, the invention provides colloidal suspensions suitable for transfecting a mammalian cell comprising a nucleic acid comprising a coding sequence for a contrast agent, such as a contrast agent described above. Optional types of colloidal suspensions include one or more of the following: a macromolecule complex, a nanocapsule, a microsphere, a bead, an oil-in-water emulsions, a micelle, a mixed micelle, and a liposomes.

In yet further aspects, the invention provides cells, cell cultures, organized cell cultures, tissues, organs and non-human organisms comprising a recombinant nucleic acid comprising a coding sequence for a contrast agent, such as a contrast agent described above. In certain embodiments, the organism is selected from the group consisting of: a mouse, a rat, a dog, a monkey, a pig, a fruit fly, a nematode worm and a fish, or alternatively a plant or fungus. In further embodiments, the cells, cell cultures, organized cell cultures, tissues, organs and non-human organisms may comprise a vector as described above.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986);

Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

The claims provided below are hereby incorporated into this section by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Correlation between ferritin increase and $1/T_1$ (a) and $1/T_2$ (b) in simulated tumors. The solid line is least-squares fit through the data (guide for the eye). The values are normalized to give the ferritin increase over the mean baseline value of the control pellet, which is 1.5 mg/ml of ferritin; the experimental samples were incubated with various concentration of ferric ammonium citrate (FAC) and the control samples were incubated in the absence of FAC. The error bars represent the standard deviation for N=4 experimental runs.

Figure 2. Data showing the percent of the total number cells remaining after the 16 hour period of ferritin loading. For each FAC concentration (and control), cells before and after the incubation period were counted 3-times using a hemocytometer and the results were averaged. The error bars represent the standard deviation for the separate (N=4) incubation experiments.

Figure 3. MRI image of three simulated tumor samples. Here, (a) is the control and (b) and (c) are the samples containing a ferritin increase of 2.7 and 4, respectively. Contrast among these samples is readily apparent in this T_2-weighted image. Images were acquired simultaneously using a Bruker 7-Tesla MRI system with TE/TR=45/2000 ms, 128x128 image points, and a 1 mm-thick slice. The pellet size was approximately 4 mm in diameter.

Figure 4: MRI image through pelleted 9L glioma cells transfected with contrast proteins light (LF) and heavy (HF) chain ferritin. The sample on the left is the control (no DNA added during incubation). Image contrast is readily apparent between the two pellets. Expression of the reporter turns cells dark in the MR image. This image was acquired using an 11.7 Tesla MRI system with a standard T_2-weighted 2DFT pulse sequence. This image was acquired at 4 °C.

Figure 5: MRI image through pelleted 9L cells infected with contrast proteins light (LF) and heavy (HF) chain ferritin via an adenovirus. The sample on the left is the control (uninfected cells). Image contrast is readily apparent between the two pellets. (Note that the intense dark spots in the pellets are bubble artifacts.) This image was acquired using an 11.7 Tesla MRI system and a standard T_2-weighted 2DFT pulse sequence. This image was acquired at 4 °C.

Figure 6: Human ferritin heavy chain cDNA sequence (BC016009) (SEQ ID NO:1). The coding region is underlined.

Figure 7: Human ferritin heavy chain amino acid sequence (AAH16009) (SEQ ID NO:2).

Figure 8: Human ferritin light chain cDNA sequence (XM_050469) (SEQ ID NO:3) The coding region is underlined.

Figure 9: Human ferritin light chain amino acid sequence (XP_050469) (SEQ ID NO:4).

Figure 10: Mus musculus ferritin heavy chain cDNA sequence (NM_010239.1) (SEQ ID NO:5). The coding region is underlined.

Figure 11: Mus musculus ferritin heavy chain amino acid sequence (NP_034369.1) (SEQ ID NO:6).

Figure 12: Mus musculus ferritin light chain 1 cDNA sequence (NM_010240.1) (SEQ ID NO:7). The coding region is underlined.

Figure 13: Mus musculus ferritin light chain 1 amino acid sequence (NP_034370.1) (SEQ ID NO:8)

Figure 14: Mus musculus ferritin light chain 2 cDNA sequence (NM_008049.1) (SEQ ID NO:9). The coding region is underlined.

Figure 15: Mus musculus ferritin light chain 2 amino acid sequence (NP_032075.1) (SEQ ID NO:10)

Figure 16: Rattus norvegicus ferritin subunit H cDNA sequence (NM_012848.1) (SEQ ID NO:11). The coding region is underlined.

Figure 17: Rattus norvegicus ferritin subunit H amino acid sequence (NP_036980.1) (SEQ ID NO:12)

Figure 18: Rattus norvegicus ferritin light chain 1 cDNA sequence (NM_022500.1) (SEQ ID NO:13). The coding region is underlined.

Figure 19: Rattus norvegicus ferritin light chain 1 amino acid sequence (NP_071945.1) (SEQ ID NO:14).

Figure 20: Homo sapiens transferrin receptor cDNA sequence (NM_003234) (SEQ ID NO:15). The coding region is underlined.

Figure 21: Homo sapiens transferrin receptor amino acid sequence (NP_003225) (SEQ ID NO:16).

Figure 22: Homo sapiens transferrin receptor 2 cDNA sequence (NM_003227) (SEQ ID NO:17). The coding region is underlined.

Figure 23: Homo sapiens transferrin receptor 2 amino acid sequence (NM_003218) (SEQ ID NO:18).

Figure 24: Mus musculus transferrin receptor coding sequence (NM_011638) (SEQ ID NO:19).

Figure 25: Mus musculus transferrin receptor amino acid sequence (NP_035768) (SEQ ID NO:20).

Figure 26: Mus musculus transferrin receptor 2 nucleic acid sequence (NM_015799) (SEQ ID NO:21). The coding region is underlined.

Figure 27: Mus musculus transferrin receptor 2 amino acid sequence (NP_056614) (SEQ ID NO:22).

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

"Coding sequence" is used herein to refer to the portion of a nucleic acid that encodes a particular protein. A coding region may be interrupted by introns and other non-coding sequences that are ultimately removed prior to translation.

"Colloidal suspension" is used herein to refer to a colloidal suspension that comprises one or more nucleic acids for delivery to cells. The material in a colloidal suspension is generally designed so as to protect nucleic acids and facilitate the delivery of nucleic acids across cell membranes. Exemplary colloidal suspensions include, but are not limited to, lipid micelles, tubes, rafts, sandwiches and other lipid structures, often comprising cationic lipids. Other colloidal suspensions include nanocapsules, microbeads and small, nucleic acid-binding polymeric structures, etc.

The term "contrast agent" is used herein to refer to a molecule that generates a contrasting effect in vivo, whether the effect is direct or indirect or both. In exemplary embodiments, "contrast agent" is used interchangeably with "contrast protein" or "contrast polypeptide." In the case of a direct effector, the contrast protein will typically form a

complex that affects the relaxation times T1, T2 or T2*. Often direct contrast proteins form metalloprotein complexes. Exemplary categories of contrast proteins include, for example, metal binding proteins and/or agents that stimulate production of one or more metal-binding protein, etc. Indirect effectors include molecules that cause a cell to produce a direct contrast protein and/or modulate a functional, biochemical, and/or biophysical characteristic of a direct contrast protein, thereby creating a contrast effect. Exemplary categories of indirect effectors include, for example, proteins and/or nucleic acids that affect expression of a direct contrast protein, modulate the activity of a direct contrast protein, modulate metal binding to a metal-binding protein, modulate expression of an iron regulatory protein, and/or modulate the activity of an iron regulatory protein, etc.

The term "contrast effect", as used herein with respect to MRI, includes any alteration in the MRI signal that renders one cell or tissue detectably different from another. A contrast effect may involve effects on T1, T2 and/or T2*. In MRI, a subject containing mobile water is generally placed in a large static magnetic field. The field tends to align some of the magnetic moments (spins) of the hydrogen nuclei in the water along the field direction. The spin lattice relaxation time (T1) is the time constant for a population of nuclei placed in a magnetic field to equilibrate along the magnetic field direction. T1 is the time constant for the transfer of energy from the spin system to the environment (the lattice). The spin-spin relaxation time (T2) is the time constant for nuclei precessing at the Larmor frequency to remain in phase with each other. Alternatively, T2 is called the spin-phase memory time. This loss of phase coherence is attributed to low-frequency fluctuations of the magnetic field that are commonly due to interactions among spins. The relaxation time T2* is defined as $1/T2^* = 1/T2 + \gamma\Delta B$, where γ is the nuclear gyromagnetic ratio and ΔB is the static external magnetic field inhomogeneity.

The terms “contrast gene” or “contrast nucleic acid” are used interchangeably herein to refer to a nucleic acid comprising a coding sequence for a contrast protein.

An “externally regulated promoter” is a nucleic acid that affects transcription in response to conditions that may be provided in a controlled manner by one of skill in the art. Externally regulated promoters may be regulated by specific chemicals, such as tetracycline or IPTG, or by other conditions such as temperature, pH, oxidation state etc. that are readily controlled external to the site of transcription.

The term “Ferritin protein” is intended to include any of a group of diiron-carboxylate proteins characterized by the tendency to form a multimeric structure with bound iron and having a helix-bundle structure comprising an iron-coordinating Glu residue in a first helix and a Glu-X-X-His motif in a second. Certain ferritins maintain bound iron in a primarily Fe(III) state. Bacterioferritins tend to be haem proteins. Vertebrate ferritins tend to be assembled from two or more subunits, and mammalian ferritins are often assembled from a heavy chain and a light chain. Many ferritins form hollow structures with an iron-rich aggregate in the interior. Exemplary ferritins are presented in Table 1 below.

"Homology" or "identity" or "similarity" refers to sequence similarity between two polypeptides or between two nucleic acid molecules. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. A sequence which is "unrelated" or "non-homologous" shares less

than 40% identity, though preferably less than 25% identity with a sequence of the present invention.

The term "homology" describes a mathematically based comparison of sequence similarities which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention may be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used. See http://www.ncbi.nlm.nih.gov.

As used herein, "identity" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in

Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990).

The term "iron binding protein" as used herein is intended to include proteins that bind to iron under physiologically relevant conditions. Certain iron binding proteins interact with iron through a cofactor such as heme. Many other exemplary cofactors are also described herein. Other iron binding proteins form an iron binding site with the appropriate amino acids, including but not limited to, histidine, aspartate, glutamate, asparagine and glutamine. Although iron binding proteins of the invention bind iron, they are also likely to bind to other metals. Accordingly, "iron binding protein" as used herein is not meant to indicate that the protein binds iron exclusively, or even that the protein binds iron more tightly than other metals.

An "iron regulatory protein" refers to a protein that is involved in iron utilization, processing, and/or accumulation in a cell. Iron regulatory proteins include, for example, proteins that regulate iron homeostasis, proteins that regulate iron trafficking into or out of a cell, proteins involved in regulating the production of iron related elements, such as, for

example, ferritin and transferrins, etc. Iron regulatory proteins may or may not bind iron directly.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include analogs of either RNA or DNA made from nucleotide analogs (including analogs with respect to the base and/or the backbone, for example, peptide nucleic acids, locked nucleic acids, mannitol nucleic acids etc.), and, as applicable to the embodiment being described, single-stranded (such as sense or antisense), double-stranded or higher order polynucleotides.

The term "operably linked" is used herein to refer to the relationship between a regulatory sequence and a gene. If the regulatory sequence is positioned relative to the gene such that the regulatory sequence is able to exert a measurable effect on the amount of gene product produced, then the regulatory sequence is operably linked to the gene.

A "polylinker" is a nucleic acid comprising at least two, and preferably three, four or more restriction sites for cleavage by one or more restriction enzymes. The restriction sites may be overlapping. Each restriction sites is preferably five, six, seven, eight or more nucleotides in length.

A "recombinant helper nucleic acid" or more simply "helper nucleic acid" is a nucleic acid which encodes functional components that allow a second nucleic acid to be encapsidated in a capsid. Typically, in the context of the present invention, the helper plasmid, or other nucleic acid, encodes viral functions and structural proteins which allow a recombinant viral vector to be encapsidated into a capsid. In one preferred embodiment, a recombinant adeno-associated virus (AAV) helper nucleic acid is a plasmid encoding AAV polypeptides, and lacking the AAV ITR regions. For example, in one embodiment, the helper plasmid encodes the AAV genome, with the exception of the AAV ITR regions,

which are replaced with adenovirus ITR sequences. This permits replication and encapsidation of the AAV replication defective recombinant vector, while preventing generation of wild-type AAV virus, e.g., by recombination.

A "regulatory nucleic acid" or "regulatory sequence" includes any nucleic acid that can exert an effect on the transcription of an operably linked open reading frame. A regulatory nucleic acid may be a core promoter, an enhancer or repressor element, a complete transcriptional regulatory region or a functional portion of any of the preceding. Mutant versions of the preceding may also be considered regulatory nucleic acids.

A "transcriptional fusion" is a nucleic acid construct that causes the expression of an mRNA comprising at least two coding regions. In other words, two or more open reading frames may be organized into a transcriptional fusion such that both open reading frames will be expressed as part of a single mRNA and then give rise, as specified by the host cell, to separate polypeptides. The open reading frames in a transcriptional fusion tend to be subject to the same transcriptional regulation, but the encoded polypeptides may be subject to distinct post-translational fates (eg. degradation, etc.). A "transcriptional fusion" may be contrasted with a "translational fusion" in which two or more open reading frames are connected so as to give rise to a single polypeptide. The fused polypeptides in a "translational fusion" tend to experience similar transcriptional, translational and post-translational regulation.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell, and is intended to include commonly used terms such as "infect" with respect to a virus or viral vector. The term "transduction" is generally used herein when the transfection with a nucleic acid is by viral delivery of the nucleic acid. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for

example, the transformed cell expresses a recombinant form of a polypeptide or, in the case of anti-sense expression from the transferred gene, the expression of a naturally-occurring form of the recombinant protein is disrupted.

As used herein, the term "transgene" refers to a nucleic acid sequence which has been introduced into a cell. Daughter cells deriving from a cell in which a transgene has been introduced are also said to contain the transgene (unless it has been deleted). A transgene can encode, e.g., a polypeptide, partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced. Optionally, a transgene-encoded polypeptide may be homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but may be designed to be inserted, or is inserted, into the genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene). Alternatively, a transgene can also be present in an episome. A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, (e.g. intron), that may be necessary for optimal expression of a selected coding sequence. A transgene may also contain no polypeptide coding region, but in such cases will generally direct expression of a functionally active RNA, such as an rRNA, tRNA, ribozyme, etc. A "therapeutic transgene" is a transgene that is introduced into a cell, tissue and/or organism for the purpose of altering a biological function in a manner that is beneficial to a subject.

"Transient transfection" refers to cases where exogenous nucleic acid is retained for a relatively short period of time, often when the nucleic acid does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein. A cell has been "stably transfected" with a nucleic acid construct comprising viral coding regions when the nucleic acid construct has been introduced inside

the cell membrane and the viral coding regions are capable of being inherited by daughter cells.

"Viral particle" is an assemblage of at least one nucleic acid and a coat comprising at least one viral protein. In general, viral particles for use in delivering nucleic acids to cells will retain the ability to insert the nucleic acid into a cell, but may be defective for many other functions, such as self-replication.

2. Exemplary Methods

In some aspects, the invention relates to methods for performing MRI using an intracellular contrast agent that is generated in situ via genetic instructions and made potent by the sequestering of metal atoms. The sequestered metal atoms are preferably endogenous metal atoms such as, for example, iron atoms. In certain embodiments, methods of the invention comprise contacting subject material with a nucleic acid encoding instructions for the synthesis of an intracellular contrast agent, such as a metal binding protein. In such an embodiment, upon internalization by an appropriate cell, the nucleic acid directs production of the metal binding protein which becomes potent as a contrast agent by binding to available metal atoms. In another embodiment, the methods of the invention comprise contacting subject material with a protein or nucleic acid that indirectly affects contrast, for example, by increasing the amount of metal in the cell or by affecting the expression and/or activity of a metal binding protein. Intracellular contrast agents described herein may be employed in the imaging of essentially any biological material that is capable of producing such an agent, including but not limited to: cultured cells, tissues, and living organisms ranging from unicellular organisms to multicellular organisms (e.g. humans, non-human mammals, other vertebrates, higher plants, insects, nematodes, fungi etc.). While most biological systems contain a variety of metals that have potent contrast

effects, it is understood that iron is generally the only such metal that is sufficiently concentrated to be useful in rendering an intracellular contrast agent potent. However, if desired, material to be imaged may be supplemented with exogenous metal atoms, and such protocols will preferably be optimized to reduce deleterious effects caused by the exogenous metal atoms.

In certain embodiments, the novel contrast technology described herein may be employed to investigate the regulation of gene expression in situ. For example, a nucleic acid encoding a contrast protein may be introduced into a cell, tissue, and/or subject of interest. Those cells having appropriate intracellular conditions for expression of the contrast protein may be distinguished by MRI from cells that do not produce the contrast protein. In certain embodiments, the nucleic acid encoding the contrast protein is operably linked to a constitutively active regulatory sequence. In further embodiments, the contrast protein is operably linked to a regulatory sequence so that production of the contrast protein may be regulated by application of one or more exogenously controlled conditions, such as temperature changes, concentration of an inducer or repressor, etc. In yet another embodiment, the activity of the regulatory sequence is at least partially unknown. In a further embodiment, the nucleic acid encoding a contrast protein is not operably linked to a regulatory sequence (or is operably linked to a weak promoter). This type of "promoterless" construct may be used to identify endogenous sequences that supply regulatory activity in a manner analogous to an "enhancer trap".

In certain exemplary embodiments, methods and compositions of the invention are used to monitor the expression of a transgene of interest, such as a therapeutic transgene. Subject material is contacted with both a transgene of interest, such as a therapeutic transgene, and a nucleic acid construct comprising the coding sequence for a contrast protein that is operably linked to a regulatory sequence. In one variation, production of the

transgene of interest and production of the contrast protein are both modulated by functionally similar (optionally identical) regulatory sequences. For example, if subject material has been contacted with a transgene under direction of a strong constitutive promoter, such as certain viral terminal repeat promoters, then expression of the gene encoding the contrast protein should also be under direction of the same promoter or a promoter designed to have a similar expression pattern. In some variations, the transgene of interest is introduced first, and then at a later time the nucleic acid encoding the contrast protein is introduced. In other variations, the nucleic acid encoding the contrast protein is introduced at the same time as the transgene of interest, and optionally the contrast nucleic acid and the transgene of interest are located on the same vector. In certain embodiments, the contrast nucleic acid is expressed as a transcriptional fusion with the transgene of interest. In further embodiments, the contrast gene and the transgene of interest (or a second copy thereof) may be expressed as a fusion protein. The fusion protein approach may be desirable where it is thought that the effectiveness of the therapeutic transgene is influenced by post-transcriptional regulation. Subject material may be imaged by MRI, and cells having the contrast protein may be detected and distinguished from cells that do not have the contrast protein. In preferred embodiments, the level of contrast detected by MRI will correlate with, or be indicative of, the level of expression of the transgene of interest.

In further exemplary embodiments, methods and compositions of the invention may be used to investigate the in situ regulatory activity of a regulatory sequence of interest. Subject material is contacted with a nucleic acid encoding a contrast protein, where the nucleic acid is operably linked to the regulatory sequence of interest. Once internalized within an appropriate cell, the contrast gene is expressed at a level that is regulated by the regulatory sequence of interest. In preferred embodiments, the level of contrast detected by MRI will be correlated with the level of activity of the regulatory sequence of interest. The

regulatory sequence of interest may be essentially any regulatory sequence, including but not limited to a promoter, an enhancer, an entire promoter/enhancer region, a mutated or altered form of the preceding, or one or more portions of the preceding.

In further exemplary embodiments, the methods described herein may be used to determine whether a physiologically important regulatory sequence is active in situ. For example, the p53 protein is a widely recognized regulator of cell proliferation and apoptosis that exerts its regulatory influences partly in response to DNA damage. Therefore, a construct comprising a p53-responsive regulatory sequence operably linked to a nucleic acid encoding a contrast protein would permit detection of cells, in situ, in which the p53 regulatory pathway has been activated. Similarly, methods of the invention may be employed to investigate, for example, the status of pro-proliferative signaling pathways (e.g. to identify cancerous or pre-cancerous cells), or to assess the status of inflammatory pathways (e.g. in host and/or donor tissues in or near transplanted organs), or to non-invasively image promoter activation during the course of development, etc. In view of this disclosure, one of skill in the art will be able develop myriad related methods.

An analogy may be drawn between the traditional reporter gene assays routinely performed by biologists, such as assays employing β-galactosidase (β-Gal) or green fluorescent protein (GFP), and certain embodiments of the present invention. Accordingly, certain methods of the invention may be used as an alternative for other commonly used cell-screening methods. For example, a method for assessing candidate pharmaceuticals may traditionally involve contacting the candidate pharmaceutical with a cell carrying an informative reporter gene construct. Now, the standard reporter gene may be replaced with a contrast gene, and the standard detection system may be replaced with an MRI system. While certain embodiments of the present invention may be used to substitute for traditional reporter gene assays, these traditional assays are far more limited in their utility.

For example, traditional assays use optically-based readout technologies that are ineffective in visualizing gene expression deep within intact tissue, and often require histological processing of the biological materials. By contrast, certain embodiments of the present invention employ an MRI contrast agent as a reporter gene, allowing signal readout deep within optically opaque tissues by MRI and, if desired, readouts may be obtained with little or no disruption of the biological function of the subject material.

In yet another exemplary embodiment, methods and compositions of the invention may be used to assess the distribution of a vector that has been administered to subject material. For example, a vector designed to transfect an organism may include a nucleic acid encoding a contrast protein operably linked to a suitable promoter. Optionally, a promoter will be selected to provide detectable levels of expression in a wide range of tissue types. For example, a strong constitutive promoter might be selected. The transfected biological material is imaged by MRI to identify cells that have been transfected with the vector. This exemplary method may be coupled with numerous different methods of administering the vector (e.g. introduction into an anatomical region or organ of particular interest, introduction into the circulatory system, the lymph system, etc.), and may be used to compare vector distribution and transcription levels obtained with each of these approaches. In the case of delivery systems that are targeted to a particular tissue, the exemplary methodology may be used to confirm or optimize tissue specificity. As another illustration, the present methods may be employed to optimize or develop a gene therapy protocol by allowing an investigator to determine the location and optionally the level of gene expression obtained after administration of a particular gene therapy system.

Many embodiments of the invention pertain to the generation of an artificially induced intracellular contrast agent. In many of the preceding embodiments, production of the intracellular contrast agent is achieved by introducing a nucleic acid encoding a direct

contrast protein. Generally, production of the contrast agent may be achieved by alternative methods. For example, in situ production of an intracellular contrast agent may be stimulated by introducing a nucleic acid encoding an indirect contrast agent. An indirect contrast agent may be, for example, a protein or nucleic acid that regulates iron homeostasis, regulates expression of an endogenous gene coding for a direct contrast agent, and/or regulates the activity of an endogenous protein that may act as a direct contrast agent, such as, for example, ferritin. As another example, production of the contrast agent may be provoked by contacting the subject material with a composition that elicits production of the contrast agent. For example, cells may be contacted with an agent, such as an iron source, that causes cells to produce ferritin, which is an effective contrast agent. Accordingly, it is understood that the invention encompasses agents that are not direct contrast agents and may be neither nucleic acid nor protein but which nonetheless are useful for inducing in situ production of an intracellular contrast agent.

In certain aspects, nucleic acids of the invention may be introduced into biological material by using any of a variety of vectors, whether general or organism/tissue/cell-type specific, and in combination with any of a variety of delivery systems, such as for example, liposomes, viral particles, electroporation, etc. In additional aspects, proteins of the invention may also be administered directly to cells in a variety of ways, such as liposome fusion, electroporation, attachment to a moiety that is internalized by cells, etc.

In certain embodiments where a nucleic acid encoding a contrast protein is introduced into cells, it may be desirable to have that gene active or present in the cells for only a short period of time, or optionally for a regulated period of time. If desired, a transient transfection system may be used, and preferably a vector that permits expression for, on average, fewer than one or two days. Alternatively, or in conjunction, gene expression may be controlled by using an externally regulated promoter, or as a further

example, the contrast gene or a portion thereof may be situated with respect to one or more recombination sites such that activation of a recombinase causes inactivation (or, if preferred, activation) of the nucleic acid encoding the contrast protein.

Many embodiments of the invention involve the use of nucleic acids encoding multiple contrast proteins, such as, for example, nucleic acids encoding heavy and light chains of a mammalian ferritin, or nucleic acids encoding a ferritin and a transferrin receptor.

In certain embodiments, the intracellular contrast agent will be chosen for safety in the subject material, and where the subject is a human subject, the intracellular contrast agent is preferably safe for use in humans.

3. Contrast Agents

In many aspects, as described above, methods of the invention will employ one or more contrast proteins that generate MRI contrast in vivo. The contrast protein will impart MRI contrast directly, or indirectly, by causing the cell to produce a secondary protein(s) that imparts MRI contrast. In the case of the direct effector, the contrast protein will typically form a complex that creates a change in at least one of relaxation times T1, T2, and/or T2*, where the change leads to a contrast effect during MRI. Often direct contrast proteins form metalloprotein complexes. In the case of indirect effectors, the contrast agent may be, for example, a protein or nucleic acid that regulates iron homeostasis, regulates expression of an endogenous gene coding for a direct contrast agent, and/or regulates the activity of an endogenous protein that may act as a direct contrast agent, thereby producing a contrast effect. In certain embodiments, the methods described herein may involve both direct and indirect contrast agents. In an exemplary embodiment, the methods and/or

compositions described herein comprises an indirect contrast agent that affects iron homeostasis and a direct contrast agent, such as a metal binding protein.

In aspects of the invention employing a metal-binding polypeptide as a direct contrast agent, the metal-binding protein will preferably bind to one or more metals that provide effective contrasting. A variety of metals are effective as elements of a contrasting agent, particularly those with unpaired electrons in the *d* or *f* orbitals, such as, for example, iron (Fe), cobalt (Co), manganese (Mn), nickel (Ni), gadolinium (Gd), etc. As noted above, iron is of particular interest because it is present at relatively high levels in mammals and most other organisms, and therefore, detectable accumulations of iron may be generated without the aid of exogenous iron supplementation. Accordingly, preferred metal-binding proteins of the invention are iron-binding proteins. In those embodiments employing a T2 contrast agent, the geometry of metal binding is not important, but the contrast will tend to be greater when larger amounts of metal are concentrated together. In certain preferred embodiments, the effective metal should be bound into a metal-rich aggregate, optionally a crystal-like aggregate, greater than 10 picometers in diameter, optionally greater than 100 picometers, greater than 1 nanometer, greater than 10 nanometers or greater than 100 nanometers in diameter. Alternatively the metal-rich aggregate should be in the range of 1-100 nanometers in diameter within the polypeptide complex. In a particularly preferred embodiment, the metal-rich aggregate exhibits properties of superparamagnetism. When an iron-binding polypeptide is used, it is preferable if the polypeptide retains the iron in the nontoxic Fe(III) oxidation state. Fe(II) is also an effective contrasting agent, but Fe(II) may participate in the iron-catalyzed HaberWeiss reaction that yields potentially damaging hydroxyl radicals.

In a preferred embodiment, a direct contrast protein of the invention has the following properties: rapid intracellular protein assembly and metal loading, the tendency to

promote formation of a metal-rich aggregate that has a large paramagnetic susceptibility, and the ability to retain the metal in a relatively non-toxic form (e.g. in the case of iron, the Fe(III) state).

In certain aspects, metal-binding polypeptides may also change the contrast properties of a cell by perturbing metal metabolism and stimulating the expression of endogenous metal-binding polypeptides that have contrast effects. This may also lead to an accumulation or depletion of a particular metal in the cell. For example, transient expression of high affinity iron-binding proteins may create a temporary decrease in the intracellular labile iron pool and stimulate production of transferrin receptor, thereby increasing the net iron uptake into the cell.

Although the exact binding affinity of a metal-binding protein for different metals is not critical, it is generally expected that polypeptides with a sub-nanomolar affinity for one or more effective metals may be useful, and optionally the polypeptide will have a dissociation constant less than 10^{-15} M, 10^{-20} M, or less for one or more effective metals. It is understood that many metal binding proteins will bind to more than one type of metal. For example, lactoferrin will form complexes with metals such as manganese and zinc. Ferritin-iron complexes are generally expected to contain some small (perhaps infinitesimal) amounts of other metals. In general, iron binding proteins are likely to bind to metals such as manganese, cobalt, zinc and chromium, although in vivo the concentration and abundance of iron is so much higher than these other metals that an iron binding protein will be primarily associated with iron.

Several exemplary metal-binding polypeptides of the invention are provided. This is in no way intended to be an exhaustive list, and, in view of the teachings herein, one of skill in the art will be able to identify or design other useful metal-binding polypeptides.

In certain exemplary embodiments, one or more ferritins may be used as a contrast protein. Ferritins of the invention include any of the group of diiron-carboxylate proteins characterized by the tendency to form a dimeric or multimeric structure with bound iron and having a helix-bundle structure comprising an iron-coordinating Glu residue in a first helix and a Glu-X-X-His motif in a second. Certain ferritins maintain bound iron in a primarily Fe(III) form. A list of exemplary ferritins is provided in Table 1. This list is intended to provide examples and is not intended to be comprehensive. Many known ferritins are not included, and it is understood that most vertebrate species will have a form of ferritin that can be used as a contrast agent. In view of this specification, one of ordinary skill in the art will be able to identify additional ferritin homologs. In certain embodiments, a ferritin for use as a contrasting agent should have at least 50% identity with the amino acid sequence of SEQ ID NO:2 and/or SEQ ID NO:4, and optionally at least 60%, 70%, 80%, 90%, 95%, 98%, 99% or 100% identity with the amino acid sequence of SEQ ID NO:2 and/or SEQ ID NO:4.

In many embodiments, methodologies of the invention will employ a vertebrate ferritin as a contrast agent. Vertebrate ferritins typically form a large complex that assembles in a shell to delimit a cavity where iron is accumulated in a mineral and compact form. Most mammalian ferritins are composed of two subunit types, the H- and L-chains. Typically the endogenous mRNAs for the two chains have nearly identical iron-responsive elements (IREs) close to the 5' termini that regulate ferritin translation by binding to iron-regulatory proteins (IRPs). When designing nucleic acid constructs for the ectopic expression of ferritins, it will often be desirable to omit or otherwise disrupt the IRE sequences. Contacting cultured cells with an elevated iron concentration typically causes a strong up-regulation of both the L- and H- chains, whereas treatment with iron chelating agents, such as desferrioxamine, suppresses ferritin production. Preferred ferritins of the

invention catalyze both an iron oxidation step from the Fe(II) form to the Fe(III) form and also catalyze the nucleation and growth of an iron mineral core. In the case of ferritins composed of multiple subunits, it will typically be desirable to express all subunits at a stoichiometry approximating that found in the native complexes. However, it is notable that a wide range of subunit ratios will typically be effective. For example, human H chain is capable of forming a homopolymer that binds iron. Excess ferritin resulting from overexpression is typically degraded inside the cell, and the primary decay product is hemosiderin deposits; these are also effective as contrast agents.

Table 1: Exemplary Ferritin Proteins and Nucleic Acids

Name	Amino Acid Sequence (Acc. No.)	Nucleic Acid Sequence (Acc. No.)
ferritin, heavy polypeptide 1 [Homo sapiens]	AAH16009.1	BC016009.1
ferritin, light polypeptide [Homo sapiens]	XP_050469.1	XM_050469.1
ferritin heavy chain [Mus musculus]	NP_034369.1	NM_010239.1
ferritin light chain 1 [Mus musculus]	NP_034370.1	NM_010240.1
ferritin light chain 2 [Mus musculus]	NP_032075.1	NM_008049.1
ferritin subunit H [Rattus norvegicus]	NP_036980.1	NM_012848.1
ferritin light chain 1 [Rattus norvegicus]	NP_071945.1	NM_022500.1
ferritin heavy chain [Cavia porcellus]	BAB70615.1	AB073371.1
ferritin light chain [Cavia porcellus]	AAF36408.1	AF233445_1
ferritin heavy chain [rabbit]	P25915	
ferritin light chain [rabbit]	S01239	
ferritin H subunit [Bos taurus]	BAA24818.1	AB003093.1
ferritin L subunit [Bos taurus]	BAA24819.1	AB003094.1

Name	Amino Acid Sequence (Acc. No.)	Nucleic Acid Sequence (Acc. No.)
ferritin heavy chain [Gallus gallus]	A26886	
ferritin [Canis familiaris]	AAK82992.1	AF285177.1
ferritin H chain [Macaca mulatta]	AAF98711.1	AF162481_1
ferritin heavy chain [Xenopus laevis]	FRXL	
ferritin heavy chain [Danio rerio]	AAG37837.1	AF295373_1
yolk ferritin [Paragonimus westermani]	AAG17056.1	AF188720_1
ferritin [Taenia saginata]	CAA65097.1	
26kDa ferritin subunit [Galleria mellonella]	AAG41120.1	AF142340.1
nonheme iron-containing ferritin (pfr) [Helicobacter pylori 26695]	NP_207447.1	NC_000915.1
ferritin [Glycine max]	AAL09920.1	AY049920.1

In a further embodiment, a metal binding protein of the invention is a metal scavenger, defined as a protein that binds metal with very high affinity through a siderophore. Such proteins may be used as contrast agents. While not wishing to be bound to a mechanism, it is expected that such proteins will act primarily as indirect contrast agents. For example, iron scavenging proteins expressed in a cell may scavenge and tightly bind iron from the labile iron pool within the intracellular space. Thus MRI contrast may be enhanced by a combination of the iron-bound chelate itself and the additional iron that is sequestered and stored as a result of the cell's own iron regulation mechanisms. Exemplary siderophores that may be present in metal scavenging proteins include hemoglobin, and any other agent that provides an octahederal coordination sphere for the iron, usually formed by six oxygen atoms. In general these fall into two categories: (a) catechols such as enterobactin which comprises a cyclic structure composed of three molecules of 2,3-

dihydroxy-N-benzoyl serine. Further examples include agents wherein the serine is substituted with either a glycine or a threonine. Also included herein are catechol siderophores having linear rather than cyclic structures such as pseudobactin; (b) Hydroxamates comprise a large and variable group having either cyclic or linear peptides containing various types of hydroxamic acids. Common examples include ferrichrome, ferrioxamine, and aerobactin. Further examples include plant siderophores such as phytosiderophore. Exemplary metal scavenging proteins include ferric binding proteins of the siderophilin family, such as mammalian transferrins, ovotransferin, lactoferrins, melanotransferrin, sertoli transferrin, neurotransferrin, mucosal transferrin, and bacterial transferrins, such as those found in *Haemophilus influenzae, Neisseria gonorrhoeae,* and *Neisseria meningitidis.*

In further embodiments, an iron regulatory protein (IRP) may be used as a contrast protein. IRPs are iron-regulating RNA binding proteins that modulate synthesis of proteins that function in the uptake (e.g. transferrin receptor), utilization (e.g. erythroid 5-aminolevulinate synthase) or storage (e.g. H-ferritin and L-ferritin) of iron. Proteins regulated by IRPs are encoded by mRNAs that include one or more stem-loop motifs, termed an Iron Responsive Element (IRE). Under low iron conditions, IRPs bind to IREs and modulate the stability or translation of the affected mRNA. In general, when an IRE is positioned in the 5' UTR region of an mRNA (e.g. the ferritins), the IRP blocks translation, causing decreased protein production in low iron conditions. When an IRE is positioned in the 3'UTR (e.g. transferrin receptor), the IRP typically stabilizes the mRNA, thereby increasing production of the gene product in response to low iron conditions. Mice having a targeted deletion of the gene encoding IRP2 show significant accumulations of iron in neural tissues (LaVaute et al., 2001, *Nat. Genet.* 27(2):209-14). Accordingly, manipulation of IRPs by, for example, antisense or RNAi methodologies may provide contrast effects.

IRPs of the invention will typically have the ability to bind to IREs in an iron-regulated manner. Preferred IRPs of the invention will be vertebrate IRPs such as: human IRP1 (Acc. Nos. P21399 and Z11559), human IRP2 (Acc. Nos. AAA69901 and M58511), rat IRE-BP1 (Acc. Nos. Q63270 and L23874), mouse IRE-BP1 (Acc. Nos. P28271 and X61147), chicken IRE-BP (Acc. No. Q90875 and D16150), etc. In general, it will be desirable to employ an IRP that binds to the IREs of the subject biological material, and in certain embodiments, this may be accomplished by using an IRP that is derived from the subject species. In certain aspects of the invention, a contrast protein comprises an amino acid sequence at least 60% identical to that of human IRP1 and/or IRP2, and optionally at least 70%, 80%, 90%, 95%, 98%, 99% or 100% identical.

In further aspects, a contrast protein of the invention may be one that perturbs cellular iron homeostasis. For example, a transferrin receptor protein, and/or a molecule that regulates the expression and/or function of a transferrin receptor protein, may be used as a contrast agent. Transferrin receptor mediates the receptor mediated endocytosis of the iron-carrying protein transferrin and thereby mediates cellular iron uptake. Therefore, in one embodiment of the invention, the level and/or activity of a transferrin receptor in targeted cells may be modulated so as to produce an increase in cellular iron uptake thereby causing the cell to produce ferritin. The end result will be an accumulation of excess ferritin that will yield MRI contrast. Exemplary transferrin receptors include SEQ ID Nos: 16, 18, 20 and 22. In certain aspects of the invention, a contrast protein comprises an amino acid sequence at least 60% identical to that of human transferrin receptor 1 and/or human transferrin receptor 2, and optionally at least 70%, 80%, 90%, 95%, 98%, 99% or 100% identical, and preferably retains transferrin receptor activity.

In further embodiments, contrast proteins of the invention may be engineered, by for example, employing techniques of molecular biology. For example, it is possible to modify

the structure of the subject contrast proteins for such purposes as enhancing contrast efficacy, stability (e.g., increased or decreased resistance to proteolytic degradation in vivo), antigenicity, or safety, among other characteristics. Such modified proteins can be produced, for instance, by amino acid substitution, deletion, or addition. In addition, simple variants of any of the proteins discussed herein may be obtained by conservative substitution. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur - containing = cysteine and methionine. (see, for example, Biochemistry, 2nd ed., Ed. by L. Stryer, W.H. Freeman and Co., 1981).

This invention further contemplates methods of generating sets of combinatorial mutants of the subject contrast proteins, as well as functional truncation mutants. The

purpose of screening such combinatorial libraries is to generate, for example, engineered contrast proteins with any number of desirable qualities such as those mentioned above.

There are many ways by which the library of potential engineered contrast proteins can be generated. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential contrast protein sequences. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, engineered contrast proteins can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (*see e.g.* Ruf et al., (1994) Biochemistry 33:1565-1572; Wang et al., (1994) J. Biol. Chem. 269:3095-3099; Balint et al., (1993), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by saturation mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34).

Whether a change in the amino acid sequence of a polypeptide results in a functional homologue can be readily determined by assessing the ability of the variant

polypeptide to, for example, bind the desired metal, produce sufficient MRI contrast in cells, and produce reduced cell toxicity.

In further aspects, any combination of contrast proteins may employed to obtain the desired contrast effects.

4. Constructs and Vectors

In certain aspects, the invention provides vectors and nucleic acid constructs comprising nucleic acids encoding one or more contrast agents. Other features of the vector or construct will generally be designed to supply desirable characteristics depending on how the contrast agent is to be generated and used. Exemplary desirable characteristics include but are not limited to, gene expression at a desired level, gene expression that is reflective of the expression of a different gene, easy clonability, transient or stable gene expression in subject cells, etc.

In certain aspects, it is desirable to use a vector that provides transient expression of the contrast agent. Such vectors will generally be unstable inside a cell, such that the nucleic acids necessary for expression of the contrast agent are lost after a relatively short period of time. Optionally, transient expression may be effected by stable repression. Exemplary transient expression vectors may be designed to provide gene expression for an average time of hours, days, weeks, or perhaps months. Often transient expression vectors do not recombine to integrate with the stable genome of the host. Exemplary transient expression vectors include: adenovirus-derived vectors, adeno-associated viruses, herpes simplex derived vectors, hybrid adeno-associated/herpes simplex viral vectors, influenza viral vectors, especially those based on the influenza A virus, and alphaviruses, for example the Sinbis and semliki forest viruses.

In some aspects the invention provides a vector or construct comprising a readily clonable nucleic acid encoding a contrast protein. For example, the coding sequence may be flanked by a polylinker on one or both sides. Polylinkers are useful for allowing one of skill in the art to readily insert the coding sequence in a variety of different vectors and constructs as required. In another example, the coding sequence may be flanked by one or more recombination sites. A variety of commercially available cloning systems use recombination sites to facilitate movement of the desired nucleic acid into different vectors. For example, the Invitrogen Gateway™ technology utilizes a phage lambda recombinase enzyme to recombine target nucleic acids with a second nucleic acid. Each nucleic acid is flanked with appropriate lambda recognition sequence, such as attL or attB. In other variations, a recombinase such as topoisomerase I may be used with nucleic acids flanked by the appropriate recognition sites. For example, the Vaccinia virus topoisomerase I protein recognizes a (C/T)CCTT sequence. These recombination systems permit rapid shuffling of flanked cassettes from one vector to another as needed. A construct or vector may include both flanking polylinkers and flanking recombination sites, as desired.

In certain aspects, the contrast gene is operably linked to a promoter. The promoter may for example, be a strong or constitutive promoter, such as the early and late promoters of SV40, or adenovirus or cytomegalovirus immediate early promoter. Optionally it may be desirable to use an externally regulated promoter, such as a tet promoter, IPTG-regulated promoters (GAL4, Plac), or the trp system. In view of this specification, one of skill in the art will readily identify other useful promoters depending on the downstream use. For example, the invention may utilize exemplary promoters such as the T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the

promoters of the yeast a-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. In addition, as noted above, it may be desirable to have a contrast gene operably linked to a promoter that provides useful information about the condition of the cell in which it is situated. In certain embodiments, it is anticipated that it will be desirable to achieve a concentration of contrast protein within target cells that permits detection above background noise, and with certain detection systems this will translate into a protein concentration of at least 1 nM or at least 10 nM.

Vectors of the invention may be essentially any nucleic acid designed to introduce and/or maintain a contrast gene in a cell or virus. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) may be used. Other vector systems suitable for gene therapy are described below.

5. Cells, Organized Cell Cultures, and Tissues

In many aspects, the invention provides cells, organized cell cultures, and tissues comprising a nucleic acid that encodes a contrast agent. Methods for generating transformed or transfected cells are widely known in the art, and it is anticipated that methods described herein may be used with essentially any cell type of interest, including but not limited to bacterial, fungal, plant and animal cells. Preferred embodiments of the invention employ mammalian cells. Cells of particular interest may include transformed

cells or other cells that either are part of a tumor or are useful as a model for cancer in vitro, stem or progenitor cells, and cells prepared for a cell therapy for a patient. Cells of the invention may be cultured cells, cell lines, cells situated in tissues and/or cells that are part of an organism.

It is further anticipated that cells may be used to generate organized cell cultures (i.e. cell cultures developing a non-random structure) and to generate organs or organ-like structures for transplant into subjects. It may be useful to non-invasively monitor some aspect of gene expression in such cells, or to otherwise provide MRI contrast in such cells. For example, muscle progenitor cells may be used to develop muscle-like organs for administration to injured muscle or for administration as a packet of cells that produce a therapeutic protein (*see e.g.* US Patent Nos. 5,399,346; 6,207,451; 5,538,722). Other cell culture methods have been used to produce neural, pancreatic, liver and many other organ types for transplant (*see e.g.* US Patent Nos. 6,146,889; 6,001,647; 5,888,705; 5,851,832 and PCT publication nos. WO 00/36091; WO 01/53461; WO 01/21767). Cells of this nature may be stably transfected with a contrast gene at an early stage of culture, or the organized culture may be transiently or stably transfected at a later point in culture to assess some aspect of cell function. Transfected cells may be administered to subjects in order to deliver a gene product, and this methodology is effective as an ex vivo gene therapy or cell therapy method. A nucleic acid encoding a contrast protein may be introduced into such cells and administered to a subject in order to monitor gene expression or viability of the administered cells. Cells transfected with the gene adenosine deaminase have been delivered to patients as an ex vivo gene therapy cure for Severe Combined Immunodeficiency Syndrome (SCID) (Cavazzana-Calvo et al., 2000, *Science* 288(5466):669-72).

6. Nucleic acids for delivery to organisms and in vitro tissues

Instead of ex vivo modification of cells, in many situations one may wish to modify cells in vivo. For this purpose, various techniques have been developed for modification of target tissue and cells in vivo. A number of viral vectors have been developed, such as described above, which allow for transfection and, in some cases, integration of the virus into the host. See, for example, Dubensky et al. (1984) Proc. Natl. Acad. Sci. USA 81, 7529-7533; Kaneda et al., (1989) Science 243,375-378; Hiebert et al. (1989) Proc. Natl. Acad. Sci. USA 86, 3594-3598; Hatzoglu et al. (1990) J. Biol. Chem. 265, 17285-17293 and Ferry, et al. (1991) Proc. Natl. Acad. Sci. USA 88, 8377-8381. The vector may be administered by injection, e.g. intravascularly or intramuscularly, inhalation, or other parenteral mode. Non-viral delivery methods such as administration of the DNA via complexes with liposomes or by injection, catheter or biolistics may also be used. Generally, in human subjects, it will be preferable to design the nucleic acid and/or the delivery system to provide transient expression of the nucleic acid encoding the contrast agent.

In general, the manner of introducing the nucleic acid will depend on the nature of the tissue, the efficiency of cellular modification required, the number of opportunities to modify the particular cells, the accessibility of the tissue to the nucleic acid composition to be introduced, and the like. The DNA introduction need not result in integration. In fact, non-integration often results in transient expression of the introduced DNA, and transient expression is often sufficient or even preferred.

Any means for the introduction of polynucleotides into mammals, human or non-human, may be adapted to the practice of this invention for the delivery of the various constructs of the invention into the intended recipient. In one embodiment of the invention, the nucleic acid constructs are delivered to cells by transfection, i.e., by delivery of "naked"

nucleic acid or in a complex with a colloidal dispersion system. A colloidal system includes macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system of this invention is a lipid-complexed or liposome-formulated DNA. In the former approach, prior to formulation of DNA, e.g., with lipid, a plasmid containing a transgene bearing the desired DNA constructs may first be experimentally optimized for expression (e.g., inclusion of an intron in the 5' untranslated region and elimination of unnecessary sequences (Felgner, et al., Ann NY Acad Sci 126-139, 1995). Formulation of DNA, e.g. with various lipid or liposome materials, may then be effected using known methods and materials and delivered to the recipient mammal. See, e.g., Canonico et al, Am J Respir Cell Mol Biol 10:24-29, 1994; Tsan et al, Am J Physiol 268; Alton et al., Nat Genet. 5:135-142, 1993 and U.S. patent No. 5,679,647 by Carson et al.

Optionally, liposomes or other colloidal dispersion systems are targeted. Targeting can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs, which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association

with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand. A certain level of targeting may be achieved through the mode of administration selected.

In certain variants of the invention, the nucleic acid constructs are delivered to cells, and particularly cells in an organism or a cultured tissue, using viral vectors. The transgene may be incorporated into any of a variety of viral vectors useful in gene therapy, such as recombinant retroviruses, adenovirus, adeno-associated virus (AAV), herpes simplex derived vectors, hybrid adeno-associated/herpes simplex viral vectors, influenza viral vectors, especially those based on the influenza A virus, and alphaviruses, for example the Sinbis and semliki forest viruses, or recombinant bacterial or eukaryotic plasmids. The following additional guidance on the choice and use of viral vectors may be helpful to the practitioner. As described in greater detail below, such embodiments of the subject expression constructs are specifically contemplated for use in various in vivo and ex vivo gene therapy protocols.

A. Herpes Virus Systems

A variety of herpes virus-based vectors have been developed for introduction of genes into mammals. For example, herpes simplex virus type 1 (HSV-1) is a human neurotropic virus of particular interest for the transfer of genes to the nervous system. After infection of target cells, herpes viruses often follow either a lytic life cycle or a latent life cycle, persisting as an intranuclear episome. In most cases, latently infected cells are not rejected by the immune system. For example, neurons latently infected with HSV-1 function normally and are not rejected. Some herpes viruses possess cell-type specific promoters that are expressed even when the virus is in a latent form.

A typical herpes virus genome is a linear double stranded DNA molecule ranging from 100 to 250 kb. HSV-1 has a 152 kb genome. The genome may include long and short

regions (termed UL and US, respectively) which are linked in either orientation by internal repeat sequences (IRL and IRS). At the non-linker end of the unique regions are terminal repeats (TRL and TRS). In HSV-1, roughly half of the 80-90 genes are non-essential, and deletion of non-essential genes creates space for roughly 40-50 kb of foreign DNA (Glorioso et al, 1995). Two latency active promoters which drive expression of latency activated transcripts have been identified and may prove useful for vector transgene expression (Marconi et al, 1996).

HSV-1 vectors are available in amplicons and recombinant HSV-1 virus forms. Amplicons are bacterially produced plasmids containing OriC, an Escherichia coli origin of replication, OriS (the HSV-1 origin of replication), HSV-1 packaging sequence, the transgene under control of an immediate-early promoter & a selectable marker (Federoff et al, 1992). The amplicon is transfected into a cell line containing a helper virus (a temperature sensitive mutant) which provides all the missing structural and regulatory genes in trans. More recent amplicons include an Epstein-Barr virus derived sequence for plasmid episomal maintenance (Wang & Vos, 1996). Recombinant viruses are made replication deficient by deletion of one the immediate-early genes e.g. ICP4, which is provided in trans. Deletion of a number of immediate-early genes substantially reduces cytotoxicity and allows expression from promoters that would be silenced in the wild type latent virus. These promoters may be of use in directing long term gene expression. Replication-conditional mutants replicate in permissive cell lines. Permissive cell lines supply a cellular enzyme to complement for a viral deficiency. Mutants include thymidine kinase (During et al, 1994), ribonuclease reductase (Kramm et al, 1997), UTPase, or the neurovirulence factor g34.5 (Kesari et al, 1995). These mutants are particularly useful for the treatment of cancers, killing the neoplastic cells which proliferate faster than other cell types (Andreansky et al, 1996, 1997). A replication-restricted HSV-1 vector has been used

to treat human malignant mesothelioma (Kucharizuk et al, 1997). In addition to neurons, wild type HSV-1 can infect other non-neuronal cell types, such as skin (Al-Saadi et al, 1983), and HSV-derived vectors may be useful for delivering transgenes to a wide array of cell types. Other examples of herpes virus vectors are known in the art (U.S. Patent No. 5,631,236 and WO 00/08191).

B. Adenoviral vectors

A viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. Knowledge of the genetic organization of adenovirus, a 36 kB, linear and double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 8 kB. In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. In addition, adenoviral vector-mediated transfection of cells is often a transient event. A combination of immune response and promoter silencing appears to limit the time over which a transgene introduced on an adenovirus vector is expressed.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. The virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, adenovirus is easy to grow and manipulate and exhibits broad host range in vitro and in vivo. This group of viruses can be obtained in high titers, e.g., $10^9 - 10^{11}$ plaque-forming unit (PFU)/ml, and they are highly infective. Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to

other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) Cell 16:683; Berkner et al., supra; and Graham et al., in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted polynucleotide of the invention can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the viral E3 promoter, or exogenously added promoter sequences.

The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) BioTechniques 6:616; Rosenfeld et al., (1991) Science 252:431-434; and Rosenfeld et al., (1992) Cell 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art.

Adenoviruses can be cell type specific, i.e., infect only restricted types of cells and/or express a transgene only in restricted types of cells. For example, the viruses may be engineered to comprise a gene under the transcriptional control of a transcription initiation region specifically regulated by target host cells, as described e.g., in U.S. Patent No. 5,698,443, by Henderson and Schuur, issued December 16, 1997. Thus, replication competent adenoviruses can be restricted to certain cells by, e.g., inserting a cell specific response element to regulate a synthesis of a protein necessary for replication, e.g., E1A or E1B.

DNA sequences of a number of adenovirus types are available from Genbank. For example, human adenovirus type 5 has GenBank Accession No.M73260. The adenovirus

DNA sequences may be obtained from any of the 42 human adenovirus types currently identified. Various adenovirus strains are available from the American Type Culture Collection, Rockville, Maryland, or by request from a number of commercial and academic sources. A transgene as described herein may be incorporated into any adenoviral vector and delivery protocol, by restriction digest, linker ligation or filling in of ends, and ligation.

Adenovirus producer cell lines can include one or more of the adenoviral genes E1, E2a, and E4 DNA sequence, for packaging adenovirus vectors in which one or more of these genes have been mutated or deleted are described, e.g., in PCT/US95/15947 (WO 96/18418) by Kadan et al.; PCT/US95/07341 (WO 95/346671) by Kovesdi et al.; PCT/FR94/00624 (WO94/28152) by Imler et al.;PCT/FR94/00851 (WO 95/02697) by Perrocaudet et al., PCT/US95/14793 (WO96/14061) by Wang et al.

C. AAV Vectors

Yet another viral vector system useful for delivery of the subject polynucleotides is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., Curr. Topics in Micro. and Immunol. (1992) 158:97-129).

AAV has not been associated with the cause of any disease. AAV is not a transforming or oncogenic virus. AAV integration into chromosomes of human cell lines does not cause any significant alteration in the growth properties or morphological characteristics of the cells. These properties of AAV also recommend it as a potentially useful human gene therapy vector.

AAV is also one of the few viruses that may integrate its DNA into non-dividing cells, e.g., pulmonary epithelial cells, and exhibits a high frequency of stable integration (see for example Flotte et al., (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et

al., (1989) J. Virol. 63:3822-3828; and McLaughlin et al., (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) PNAS USA 81:6466-6470; Tratschin et al., (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al., (1988) Mol. Endocrinol. 2:32-39; Tratschin et al., (1984) J. Virol. 51:611-619; and Flotte et al., (1993) J. Biol. Chem. 268:3781-3790).

The AAV-based expression vector to be used typically includes the 145 nucleotide AAV inverted terminal repeats (ITRs) flanking a restriction site that can be used for subcloning of the transgene, either directly using the restriction site available, or by excision of the transgene with restriction enzymes followed by blunting of the ends, ligation of appropriate DNA linkers, restriction digestion, and ligation into the site between the ITRs. The capacity of AAV vectors is usually about 4.4 kb (Kotin, R.M., Human Gene Therapy 5:793-801, 1994 and Flotte, et al. J. Biol.Chem. 268:3781-3790, 1993).

AAV stocks can be produced as described in Hermonat and Muzyczka (1984) PNAS 81:6466, modified by using the pAAV/Ad described by Samulski et al. (1989) J. Virol. 63:3822. Concentration and purification of the virus can be achieved by reported methods such as banding in cesium chloride gradients, as was used for the initial report of AAV vector expression in vivo (Flotte, et al. J.Biol. Chem. 268:3781-3790, 1993) or chromatographic purification, as described in O'Riordan et al., WO97/08298. Methods for in vitro packaging AAV vectors are also available and have the advantage that there is no size limitation of the DNA packaged into the particles (see, U.S. Patent No. 5,688,676, by

Zhou et al., issued Nov. 18, 1997). This procedure involves the preparation of cell free packaging extracts.

D. ***Hybrid Adenovirus-AAV Vectors***

Hybrid Adenovirus-AAV vectors have been generated and are typically represented by an adenovirus capsid containing a nucleic acid comprising a portion of an adenovirus, and 5' and 3' inverted terminal repeat sequences from an AAV which flank a selected transgene under the control of a promoter. See e.g. Wilson et al, International Patent Application Publication No. WO 96/13598. This hybrid vector is characterized by high titer transgene delivery to a host cell and the ability to stably integrate the transgene into the host cell chromosome in the presence of the rep gene. This virus is capable of infecting virtually all cell types (conferred by its adenovirus sequences) and stable long term transgene integration into the host cell genome (conferred by its AAV sequences).

The adenovirus nucleic acid sequences employed in this vector can range from a minimum sequence amount, which requires the use of a helper virus to produce the hybrid virus particle, to only selected deletions of adenovirus genes, which deleted gene products can be supplied in the hybrid viral process by a packaging cell. For example, a hybrid virus can comprise the 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as origins of replication). The left terminal sequence (5') sequence of the Ad5 genome that can be used spans bp 1 to about 360 of the conventional adenovirus genome (also referred to as map units 0-1) and includes the 5' ITR and the packaging/enhancer domain. The 3' adenovirus sequences of the hybrid virus include the right terminal 3' ITR sequence which is about 580 nucleotides (about bp 35,353- end of the adenovirus, referred to as about map units 98.4-100).

For additional detailed guidance on adenovirus and hybrid adenovirus-AAV technology which may be useful in the practice of the subject invention, including methods

and materials for the incorporation of a transgene, the propagation and purification of recombinant virus containing the transgene, and its use in transfecting cells and mammals, see also Wilson et al, WO 94/28938, WO 96/13597 and WO 96/26285, and references cited therein.

E. Retroviruses

In order to construct a retroviral vector, a nucleic acid of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and psi components is constructed (Mann et al. (1983) Cell 33:153). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and psi sequences is introduced into this cell line (by calcium phosphate precipitation for example), the psi sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein (1988) "Retroviral Vectors", In: Rodriguez and Denhardt ed. Vectors: A Survey of Molecular Cloning Vectors and their Uses. Stoneham:Butterworth; Temin, (1986) "Retrovirus Vectors for Gene Transfer: Efficient Integration into and Expression of Exogenous DNA in Vertebrate Cell Genome", In: Kucherlapati ed. Gene Transfer. New York: Plenum Press; Mann et al., 1983, supra). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. Integration and stable expression require the division of host cells (Paskind et al. (1975) Virology 67:242). This aspect is particularly relevant for the treatment of PVR, since these vectors allow selective targeting of cells which proliferate, i.e., selective targeting of the cells in the epiretinal membrane, since these are the only ones proliferating in eyes of PVR subjects.

A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding a protein of the present invention, e.g., a transcriptional activator, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. A preferred retroviral vector is a pSR MSVtkNeo (Muller et al. (1991) Mol. Cell Biol. 11:1785 and pSR MSV(XbaI) (Sawyers et al. (1995) J. Exp. Med. 181:307) and derivatives thereof. For example, the unique BamHI sites in both of these vectors can be removed by digesting the vectors with BamHI, filling in with Klenow and religating to produce pSMTN2 and pSMTX2, respectively, as described in PCT/US96/09948 by Clackson et al. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am.

Retroviruses, including lentiviruses, have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, retinal cells,

endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example, review by Federico (1999) Curr. Opin. Biotechnol. 10:448; Eglitis et al., (1985) Science 230:1395-1398; Danos and Mulligan, (1988) PNAS USA 85:6460-6464; Wilson et al., (1988) PNAS USA 85:3014-3018; Armentano et al., (1990) PNAS USA 87:6141-6145; Huber et al., (1991) PNAS USA 88:8039-8043; Ferry et al., (1991) PNAS USA 88:8377-8381; Chowdhury et al., (1991) Science 254:1802-1805; van Beusechem et al., (1992) PNAS USA 89:7640-7644; Kay et al., (1992) Human Gene Therapy 3:641-647; Dai et al., (1992) PNAS USA 89:10892-10895; Hwu et al., (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al., (1989) PNAS USA 86:9079-9083; Julan et al., (1992) J. Gen Virol 73:3251-3255; and Goud et al., (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et al., (1991) J. Biol. Chem. 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/env fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

F. Other Viral Systems

Other viral vector systems that can be used to deliver a polynucleotide of the invention have been derived from vaccinia virus, alphavirus, poxvirus, arena virus, polio virus, and the like. Such vectors offer several attractive features for various mammalian cells. (Ridgeway (1988) In: Rodriguez R L, Denhardt D T, ed. Vectors: A survey of molecular cloning vectors and their uses. Stoneham: Butterworth; Baichwal and Sugden (1986) In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press; Coupar et al. (1988) Gene, 68:1-10; Walther and Stein (2000) Drugs 60:249-71; Timiryasova et al. (2001) J Gene Med 3:468-77; Schlesinger (2001) Expert Opin Biol Ther 1:177-91; Khromykh (2000) Curr Opin Mol Ther 2:555-69; Friedmann (1989) Science, 244:1275-1281 ; Ridgeway, 1988, supra; Baichwal and Sugden, 1986, supra; Coupar et al., 1988; Horwich et al.(1990) J.Virol., 64:642-650).

7. Transgenic animals

While the techniques described herein may be used to deliver nucleic acids to human or animal subjects, other methods are available to generate non-human transgenic animals incorporating a recombinant nucleic acid encoding a contrast protein.

In an exemplary embodiment, the "transgenic non-human animals" of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used to practice this invention are selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness. In addition, the haplotype is a significant factor. For example, when transgenic mice are to be produced, strains such as C57BL/6 or FVB lines are often

used (Jackson Laboratory, Bar Harbor, ME). Preferred strains such as C57BL/6 or DBA/1 may be selected. The line(s) used to practice this invention may themselves be transgenics, and/or may be knockouts (i.e., obtained from animals which have one or more genes partially or completely suppressed).

In one embodiment, the construct comprising a nucleic acid encoding a contrast protein is introduced into a single stage embryo. The zygote is the best target for microinjection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2 pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster et al. (1985) PNAS 82:4438-4442). As a consequence, all cells of the transgenic animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene.

Normally, fertilized embryos are incubated in suitable media until the pronuclei appear. At about this time, the nucleotide sequence comprising the transgene is introduced into the female or male pronucleus as described below. In some species such as mice, the male pronucleus is preferred. It is most preferred that the exogenous genetic material be added to the male DNA complement of the zygote prior to its being processed by the ovum nucleus or the zygote female pronucleus. It is thought that the ovum nucleus or female pronucleus release molecules which affect the male DNA complement, perhaps by replacing the protamines of the male DNA with histones, thereby facilitating the combination of the female and male DNA complements to form the diploid zygote.

Thus, it is preferred that the exogenous genetic material be added to the male complement of DNA or any other complement of DNA prior to its being affected by the

female pronucleus. For example, the exogenous genetic material is added to the early male pronucleus, as soon as possible after the formation of the male pronucleus, which is when the male and female pronuclei are well separated and both are located close to the cell membrane. Alternatively, the exogenous genetic material could be added to the nucleus of the sperm after it has been induced to undergo decondensation. Sperm containing the exogenous genetic material can then be added to the ovum or the decondensed sperm could be added to the ovum with the transgene constructs being added as soon as possible thereafter.

Introduction of the transgene nucleotide sequence into the embryo may be accomplished by any means known in the art such as, for example, microinjection, electroporation, or lipofection. Following introduction of the transgene nucleotide sequence into the embryo, the embryo may be incubated in vitro for varying amounts of time, or reimplanted into the surrogate host, or both. In vitro incubation to maturity is within the scope of this invention. One common method in to incubate the embryos in vitro for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

For the purposes of this invention a zygote is essentially the formation of a diploid cell which is capable of developing into a complete organism. Generally, the zygote will be comprised of an egg containing a nucleus formed, either naturally or artificially, by the fusion of two haploid nuclei from a gamete or gametes. Thus, the gamete nuclei must be ones which are naturally compatible, i.e., ones which result in a viable zygote capable of undergoing differentiation and developing into a functioning organism. Generally, a euploid zygote is preferred. If an aneuploid zygote is obtained, then the number of chromosomes should not vary by more than one with respect to the euploid number of the organism from which either gamete originated.

In addition to similar biological considerations, physical ones also govern the amount (e.g., volume) of exogenous genetic material which can be added to the nucleus of the zygote or to the genetic material which forms a part of the zygote nucleus. If no genetic material is removed, then the amount of exogenous genetic material which can be added is limited by the amount which will be absorbed without being physically disruptive. Generally, the volume of exogenous genetic material inserted will not exceed about 10 picoliters. The physical effects of addition must not be so great as to physically destroy the viability of the zygote. The biological limit of the number and variety of DNA sequences will vary depending upon the particular zygote and functions of the exogenous genetic material and will be readily apparent to one skilled in the art, because the genetic material, including the exogenous genetic material, of the resulting zygote must be biologically capable of initiating and maintaining the differentiation and development of the zygote into a functional organism.

The number of copies of the transgene constructs which are added to the zygote is dependent upon the total amount of exogenous genetic material added and will be the amount which enables the genetic transformation to occur. Theoretically only one copy is required; however, generally, numerous copies are utilized, for example, 1,000-20,000 copies of the transgene construct, in order to insure that one copy is functional. As regards the present invention, there will often be an advantage to having more than one functioning copy of each of the inserted exogenous DNA sequences to enhance the phenotypic expression of the exogenous DNA sequences.

Any technique which allows for the addition of the exogenous genetic material into nucleic genetic material can be utilized so long as it is not destructive to the cell, nuclear membrane or other existing cellular or genetic structures. The exogenous genetic material is

preferentially inserted into the nucleic genetic material by microinjection. Microinjection of cells and cellular structures is known and is used in the art.

Reimplantation is accomplished using standard methods. Usually, the surrogate host is anesthetized, and the embryos are inserted into the oviduct. The number of embryos implanted into a particular host will vary by species, but will usually be comparable to the number of off spring the species naturally produces.

Transgenic offspring of the surrogate host may be screened for the presence and/or expression of the transgene by any suitable method. Screening is often accomplished by Southern blot or Northern blot analysis, using a probe that is complementary to at least a portion of the transgene. Western blot analysis using an antibody against the protein encoded by the transgene may be employed as an alternative or additional method for screening for the presence of the transgene product. Typically, DNA is prepared from tail tissue and analyzed by Southern analysis or PCR for the transgene. Alternatively, the tissues or cells believed to express the transgene at the highest levels are tested for the presence and expression of the transgene using Southern analysis or PCR, although any tissues or cell types may be used for this analysis.

Alternative or additional methods for evaluating the presence of the transgene include, without limitation, suitable biochemical assays such as enzyme and/or immunological assays, histological stains for particular marker or enzyme activities, flow cytometric analysis, and the like. Analysis of the blood may also be useful to detect the presence of the transgene product in the blood, as well as to evaluate the effect of the transgene on the levels of various types of blood cells and other blood constituents. Alternatively, MRI can be used to visualize transgene expression.

An alternative method for generating transgenic animals involves the in vivo or ex vivo (in vitro) transfection of male animal germ cells with a desired nucleic acid (see e.g.,

U.S. Pat. No. 6,316,692). In one approach, the nucleic acid is delivered in situ to the gonad of the animal (in vivo transfection). The transfected germ cells are allowed to differentiate in their own milieu, and then animals exhibiting integration of the nucleic acid into the germ cells are selected. The selected animals may be mated, or their sperm utilized for insemination or in vitro fertilization to produce transgenic progeny. The selection may take place after biopsy of one or both gonads, or after examination of the animal's ejaculate to confirm the incorporation of the desired nucleic acid sequence. Alternatively, male germ cells may be isolated from a donor animal and transfected, or genetically altered in vitro. Following this genetic manipulation, transfected germ cells are selected and transferred to the testis of a suitable recipient animal. Before transfer of the germ cells, the recipient testis are generally treated in one, or a combination, of a number of ways to inactivate or destroy endogenous germ cells, including by gamma irradiation, by chemical treatment, by means of infectious agents such as viruses, or by autoimmune depletion or by combinations thereof. This treatment facilitates the colonization of the recipient testis by the altered donor cells. Animals that carry suitably modified sperm cells may be allowed to mate naturally, or alternatively their spermatozoa are used for insemination or in vitro fertilization.

In an exemplary embodiment, a transgenic animal may be produced by in vitro infection of a single-cell embryo with a lentiviral vector. See e.g., Lois et al., Science 295: 868-872 (2002).

Retroviral infection can also be used to introduce the transgene into a non-human animal. The developing non-human embryo can be cultured in vitro to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, R. (1976) PNAS 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Manipulating the Mouse Embryo, Hogan eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986). The viral vector system

used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al. (1985) PNAS 82:6927-6931; Van der Putten et al. (1985) PNAS 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart et al. (1987) EMBO J. 6:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al. (1982) Nature 298:623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line by intrauterine retroviral infection of the midgestation embryo (Jahner et al. (1982) supra).

A fourth type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro and fused with embryos (Evans et al. (1981) Nature 292:154-156; Bradley et al. (1984) Nature 309:255-258; Gossler et al. (1986) PNAS 83: 9065-9069; and Robertson et al. (1986) Nature 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) Science 240:1468-1474.

In general, progeny of transgenic animals may be obtained by mating the transgenic animal with a suitable partner, or by in vitro fertilization of eggs and/or sperm obtained from the transgenic animal. Where mating with a partner is to be performed, the partner may or may not be transgenic and/or a knockout; where it is transgenic, it may contain the

same or a different transgene, or both. Alternatively, the partner may be a parental line. Where in vitro fertilization is used, the fertilized embryo may be implanted into a surrogate host or incubated in vitro, or both. Using either method, the progeny may be evaluated for the presence of the transgene using methods described above, or other appropriate methods.

The transgenic animals produced in accordance with the present invention will include exogenous genetic material encoding a contrast agent. Further, the sequence will preferably be attached to a regulatory sequence that allows the expression of the transgene. Contrast agent produced in situ may be visualized by MRI.

8. MRI Methodologies

In general, contrast agents of the invention are designed for use in MRI detection systems. In the most common implementation of MRI, one observes the hydrogen nucleus (proton) in molecules of mobile water contained in subject materials. The subject material is placed in a large static magnetic field. The field tends to align the magnetic moment associated with the hydrogen nuclei in water along the field direction. The nuclei are perturbed from equilibrium by pulsed radio-frequency (RF) radiation set at the Larmor frequency, which is a characteristic frequency proportional to the magnetic field strength where protons resonantly absorb energy. Upon removing the RF, the nuclei induce a transient voltage in a receiver antenna; this transient voltage constitutes the nuclear magnetic resonance (NMR) signal. Spatial information is encoded in both the frequency and/or phase of the NMR signal by selective application of magnetic field gradients that are superimposed onto the large static field. The transient voltages are generally digitized, and then these signals may be processed by, for example, using a computer to yield images.

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration

of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

EXAMPLES

EXAMPLE 1: NMR of K562 Cells Over-Expressing Ferritin: Simulated Tumor Studies

We describe data showing the feasibility of using of an over-expression of intra-cellular metal-binding polypeptides as a potent MRI contrast agent. These initial results focus on ferritin in living human myeloid leukemia (K562) cells.

To investigate the sensitivity of ferritin in modulating the NMR properties of K562 cells, we synthesized simulated "tumor" samples. These consisted of K562 cells that were stimulated to produce varying amounts of excess intra-cellular ferritin in vitro. Cells were then suspended in low-melting point agarose to form small pellets. The spin-lattice relaxation rate ($1/T_1$) and the spin-spin relaxation rate ($1/T_2$) were measured in the pellets to quantify the impact of ferritin. (Modulation of these relaxation times give rise to image contrast in MRI.) In the same cells used for the samples, we assayed the total ferritin content using ELISA (Enzyme Linked Immuno-Sorbent Assay).

For the experiment, samples consisted of K562 cells that were stimulated to over-express ferritin by a 16 hour incubation with varying concentrations of ferric ammonium citrate (FAC) in RPMI culture media supplemented with 2% fetal calf serum. After incubation, cells were washed. For each FAC concentration, 10^7 cells were counted for the NMR sample and 10^6 cells we set aside for the ELISA assay (Alpha Diagnostics Int. Inc., San Antonio, TX)). Cells used for the NMR samples were re-suspended in 50 μl of low melting point agarose in a small plastic tube. The $1/T_1$ and $1/T_2$ measurements were performed at room temperature using a Bruker Minispec relaxometer (Bruker Instruments,

Billerica, MA). Cells used for the ELISA were treated with lysis buffer and the consistency of the total amount of released protein was confirmed using a bicinchoninic acid protein quantitation assay (Pierce Inc., Rockford, IL). Ferritin concentration was calculated as an average over the cell pellet volume.

The correlation between the NMR changes and ferritin content is shown in Figure 1. The results show substantial changes in the relaxation times with modest increases in ferritin expression over background; these changes are easily observed using MRI (below). These simulated tumors have a cell density of 200 cells/nl.

EXAMPLE 2: Toxicity Studies

The ferritin synthesis temporarily perturbs the cell's iron metabolism. Although the adverse effects of this on the cell's long-term health have yet to be fully determined in vivo, indications from various in vitro experiments have shown that ferritin overexpression is not harmful in a variety of cell lines, especially for transient expression. This was confirmed in our experiments in K562 cells described in Example 1 above. For each FAC concentration (and control), cells before and after the incubation period were counted 3-times using a hemocytometer and the results were averaged. Figure 2 shows the percent cells remaining after the 16 hour period of ferritin loading. In the simulated tumors, ferritin increases of greater than 10-times over baseline levels only resulted in a cell loss of order 20%. The ferritin increase required to provide observable MRI contrast is only of order 2-4.

EXAMPLE 3: MRI of Simulated Tumors

Ferritin over-expression in the simulated tumors is readily visualized using MRI. Figure 3 shows a MRI image slice through three pellets used in the NMR experiments. In this image, contrast is predominately T_2-weighted. In Figure 3, (a) is the control, and (b)-(c) are

the samples containing a ferritin increase of 2.7 and 4, respectively (see Fig. 1). Images were acquired simultaneously using a Bruker 7-Tesla MRI system with TE/TR=45/2000 ms, 128x128 image points, and a 1 mm-thick slice. The pellet size was approximately 4 mm in diameter.

EXAMPLE 4: MRI Studies of Cells Comprising Recombinant Ferritin

Both the light and heavy ferritin transgenes, denoted LF and HF, respectively, were introduced into variety of cell lines (e.g. K562 and Rat 9L gliosarcoma) using lipid-based transfection methods and by using viruses. The results were analyzed using ELISA, NMR, and MRI. Typical results are shown in Figures 4 and 5. Human light and heavy chain ferritin cDNA having defective iron regulatory elements were used. Using standard molecular biology techniques both transgenes were placed under the control of the immediate early promoter of the CMV. The integrity of the transgenes was confirmed by electrophoresis of DNA fragments following digestion with various restriction enzymes and by DNA sequencing.

Introduction of Ferritin via Transfection

9L cells (Fischer 344 rat gliosarcoma) were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and glutamine. Cells were plated in 24-well plates one day before transfection to achieve 60-80% confluence. The cells were rinsed with serum-free DMEM and then covered with the same solution. A DNA mixture was prepared as follows. The reagent lipofectamine™ (Invitrogen, Carlsbad, CA) was combined with equal amounts of LF and HF DNA in serum-free DMEM. The reagent Plus™ (Invitrogen, Carlsbad, CA) was added to the DNA solution to increase transfection efficiency. The DNA mixture was added to the cells, and then incubated for 3 hours at 37° C, after which DMEM containing 10% FBS was added. Cells were collected 48 or 96 hours

post-transfection and counted. In addition, control samples were prepared by incubating 9L cells under identical conditions as above, except that no DNA was added to the lipofectamine™ – Plus™ – DMEM mixture. Upon harvesting after 48 or 96 hours no significant differences in cell numbers were observed between samples incubated with the DNA reporters and the control samples. Thus, there was no apparent toxicity associated with the contrast proteins.

To assay the ferritin increase after transfection, 9L cells were prepared as described above. The intracellular proteins were extracted using the M-PER™ extraction Reagent (Pierce Biotechnology, Mountain View, CA) and the ferritin content was assayed using an ELISA kit (Alpha diagnostics, San Antonio, TX). The results typically showed a ferritin concentration ~3 ng/ml in the transfected cells and a negligible (~0.0 ng/ml) amount of human ferritin in the non-transfected cells. (The 9L cell line is from rat, and the antibody used in the ELISA detects only human ferritin with no cross-reactivity.)

The intracellular iron content was measured in transfected and control cells to confirm an increased iron-uptake with transgene expression. For these experiments $20x10^6$ cells were plated and transfected using the methods described above. Control cells were also prepared as described above with no DNA added to the incubation solution. Cells were collected 96 hours post transfection and counted. Using standard methods [2001 *Blood* 97(9), 2863] cells were washed in PBS, and pellets were dissolved in an acid solution and treated with a batophenan troline sulconate solution. The light absorption of the solution was read at 535 nm using a spectrophotometer and the iron concentration was calculated. The results indicate a factor of ~1.5 increase in the net iron content of the transfected cells compared control.

Measurement of $1/T_2$ in pellets of transfected cells was performed. Cells ($20x10^6$) were transfected with the transgenes as described above. Cells were collected 96 hours

post-transfection, washed twice with PBS, and transferred to a 0.2 ml micro-centrifuge tubes. Cells were again centrifuged and the supernatant discarded. NMR measurements were performed on the pellets at 4 ° C using a 20 MHz Bruker Minispec NMR analyzer (Bruker Instruments, Billerica, MA). The results typically show a factor of ~15 % increase in $1/T_2$ in the transfected cells over control.

Using the same cell pellets that were prepared for the above NMR experiments, we confirmed that the $1/T_2$ changes due to the expression of the contrast proteins provided satisfactory contrast in MR images. The micro-centrifuge tubes containing the pellets were placed in an MRI apparatus and imaged using a standard T_2-weighted two-dimensional Fourier transform (2DFT) spin-echo pulse sequence. Figure 4 displays typical data and shows a high-resolution MRI slice through two pellets acquired simultaneously; the left pellet is the control and the pellet on the right contains cells expressing the contrast proteins. Image contrast is clearly apparent between the two samples.

Introduction of Ferritin via a Viral Vector

Contrast proteins have also been introduced into cells via a viral vector. Infected cells were characterized using ELISA, NMR, and MRI. The MRI data shows distinct contrast between cells infected with the contrast proteins and uninfected (control) cells. For these experiments the LF and HF transgenes were each incorporated into separate replication defective adenoviruses. These viruses were constructed using the commercially available Adeno-X™ expression system (Clontech, Palo Alto, CA) following the manufacture's instructions. The transgene expression was controlled using the CMV promoter. A HEK-293 cell line was used for production of viral stocks. When the cytopathic effect was evident in the HEK-293 cells due to viral production, cells were collected, lysed, and the supernatants were collected. These supernatants are adenovirus-rich and were used to infect mammalian cells to demonstrate MRI contrasting effects. 9L cells were incubated in DMEM supplemented with 10% FBS, penicillin, streptomycin, and

glutamine. Cells (~$20x10^6$) were plated in 24-well plates one day before infection to achieve 60-80% confluence. The cells were then rinsed with serum-free DMEM and then covered with the same solution. Equal volumes of both the LF and HF adenovirus from each of the respective supernatants were added to the 9L cells. The virus and cells were incubated in serum-free media for 0.5 hour, and then FBS was added to the DMEM to give 10% FBS. After a 48 hours incubation the cells were harvested, rinsed, and the effects of the contrast genes were assayed. Figure 5 shows typical MRI data of two pellets, infected and uninfected (control), 9L cells. These data were acquired using a T_2-weighted 2DFT spin-echo sequence in a similar manner as the transfection experiments above. The left pellet is the control and the right pellet contains cells infected with LF and HF transgenes. Image contrast is clearly apparent between the two samples.

EXAMPLE 5: Introduction of a NucleicAcid Encoding a Contrast Protein *In Vivo*

This experiment is designed to demonstrate the delivery of contrast agent of the invention in vivo.

In this example, two tumor samples are transplanted onto a nude mouse. An HSV delivery is engineered to contain a nucleic acid construct comprising the coding sequences for the human ferritins represented in SEQ ID Nos: 2 and 4. One tumor sample is injected with the HSV+ferritin vector, while the other tumor sample is injected with an "empty" HSV vector. The mouse is subjected to MRI, and the contrast between the HSV+ferritin sample and the "empty" HSV sample is compared.

INCORPORATION BY REFERENCE

All of the patents, publications and sequence database entries cited herein are hereby incorporated by reference. Also incorporated by reference are the following:

Trinder et al., Int. J. Biochem. & Cell Biol., 35: 292-296 (2003); Fleming et al., Proc. Natl. Acad. Sci. USA 99: 10653-10658 (2002); and Fleming et al., Proc. Natl. Acad. Sci. USA 97: 2214-2219 (2000).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What Is Claimed:

1. A method of generating an image of a subject material comprising

providing a subject material comprising a plurality of cells wherein a subset of the cells comprise a MRI-detectable amount of contrast protein; and

imaging the cells by magnetic resonance imaging.

2. The method of claim 1, wherein the cells comprising the measurable amount of contrast protein are distinguishable from cells or other components of the material that do not comprise the measurable amount of contrast protein.

3. A method of detecting gene expression comprising

providing a cell comprising a recombinant nucleic acid encoding a contrast protein; and

imaging the cell by magnetic resonance imaging

wherein detection of the contrast protein by magnetic resonance imaging indicates that the nucleic acid encoding the contrast protein is and/or has been expressed.

4. A method of claim 3, wherein the contrast protein is a metal-binding protein.

5. A method of claim 3, wherein the contrast protein is selected from the group consisting of: a ferritin protein; a transferrin receptor protein; an iron regulatory protein; and an iron scavenger protein.

6. A method of claim 3, wherein the contrast protein is at least 60% identical to a protein selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

7. A method of claim 3, wherein the cell is part of a cell culture.

8. A method of claim 3, wherein the cell is part of an in vitro tissue.

9. A method of claim 3, wherein the cell is part of a multicellular organism.

10. A method of claim 3, wherein the cell is part of a mammal.

11. A method of claim 3, wherein the cell is part of a plant.

12. A viral particle suitable for transfecting a mammalian cell, comprising a nucleic acid comprising a coding sequence for a contrast protein.

13. The viral particle of claim 12, wherein the contrast protein is selected from the group consisting of: a ferritin protein; a transferrin receptor protein; an iron regulatory protein; and an iron scavenger protein.

14. The viral particle of claim 12, wherein the contrast protein is selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

15. The viral particle of claim 12, wherein the nucleic acid comprises a coding sequence for human L chain ferritin and for a human H-chain ferritin.

16. The viral particle of claim 12, wherein the viral particle is derived from one or more of the following: an adenovirus, an adenovirus-associated virus, a herpes simplex virus, a retrovirus, an alphavirus, a poxvirus, an arena virus, a vaccinia virus, an influenza virus and a polio virus.

17. A colloidal suspension suitable for transfecting a mammalian cell comprising a nucleic acid comprising a coding sequence for a contrast protein.

18. The colloidal suspension of claim 17, wherein the contrast protein is selected from the group consisting of: a ferritin protein; a transferrin receptor protein; an iron regulatory protein; and an iron scavenger protein.

19. The colloidal suspension of claim 17, wherein the contrast protein is selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

20. The colloidal suspension of claim 17, wherein the nucleic acid comprises a coding sequence for human L chain ferritin and for a human H-chain ferritin.

21. A colloidal suspension of claim 17, wherein the nucleic acid is complexed with one or more of the following: a macromolecule complex, a nanocapsule, a microsphere, a bead, an oil-in-water emulsions, a micelle, a mixed micelle, and a liposome.

22. A non-human multicellular organism comprising a recombinant nucleic acid comprising a coding sequence for a contrast protein.

23. The non-human multicellular organism of claim 22, wherein the contrast protein is selected from the group consisting of: a ferritin protein; a transferrin receptor protein; an iron regulatory protein; and an iron scavenger protein.

24. The non-human animal of claim 22, wherein the contrast protein is selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

25. The non-human animal of claim 22, wherein the recombinant nucleic acid comprises a coding sequence for human L chain ferritin and for a human H-chain ferritin.

26. The non-human multicellular organism of claim 22, wherein the organism is selected from the group consisting of: a mouse, a rat, a dog, a monkey, a pig, a fruit fly, a nematode worm and a fish..

27. The non-human multicellular organism of claim 22, wherein the organism is a plant.

28. A vector for transfection of a multicellular organism comprising a recombinant nucleic acid encoding a metal-binding protein, wherein the vector is an viral vector derived from one or more

of the viruses selected from the group consisting of: an adenovirus, an adenovirus-associated virus, a herpes simplex virus, a retrovirus, an alphavirus, a poxvirus, an arena virus, a vaccinia virus, an influenza virus and a polio virus.

29. The vector of claim 28, wherein the metal binding protein is selected from the group consisting of: a ferritin protein; a transferrin receptor protein; an iron regulatory protein; and an iron scavenger protein.

30. The vector of claim 28, wherein the metal binding protein is a mammalian ferritin protein.

31. The vector of claim 28, wherein the metal binding protein comprises an amino acid sequence at least 80% identical to SEQ ID NO: 2 or SEQ ID NO:4.

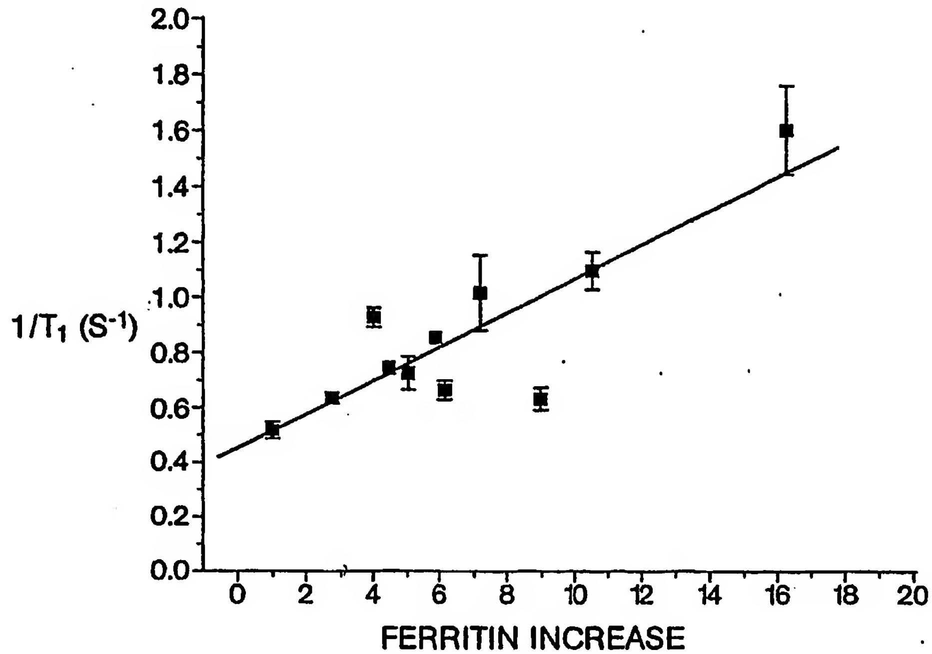
32. The vector of claim 28, wherein expression of the nucleic acid encoding the metal binding protein is regulated by a constitutive promoter.

33. A cell comprising a vector of claim 28.

34. The cell of claim 33, wherein the cell is selected from the group consisting of: a bacterial cell, a plant cell, a fungal cell, and an animal cell.

35. The cell of claim 33, wherein the cell is a mammalian cell.

36. The cell of claim 35, wherein the cell is a cancer cell.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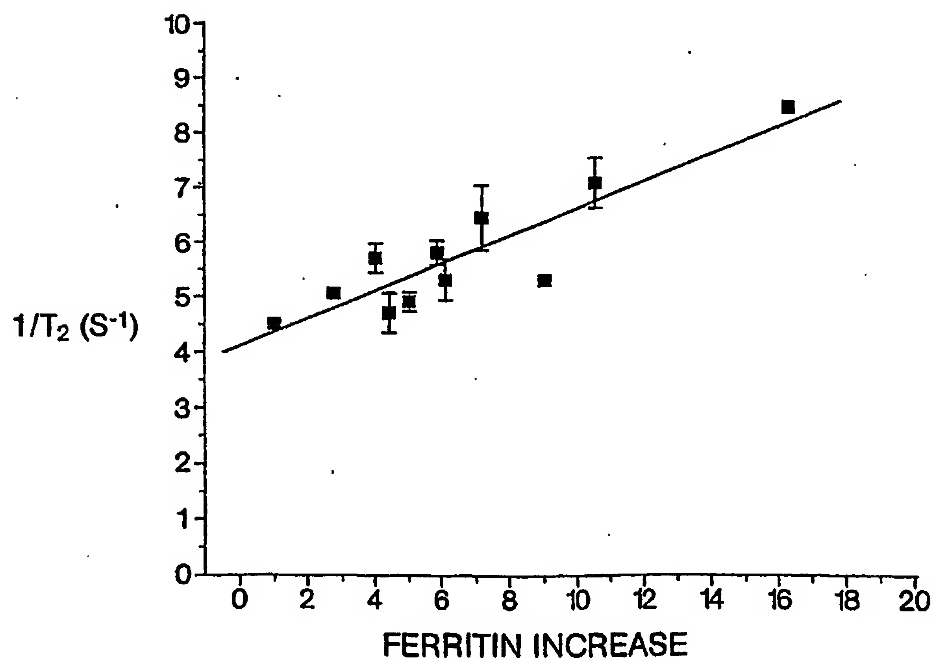
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1/T1 (S-1)
0
2
4
6
8
10
12
14
16
18
20
FERRITIN INCREASE

Fig. 1a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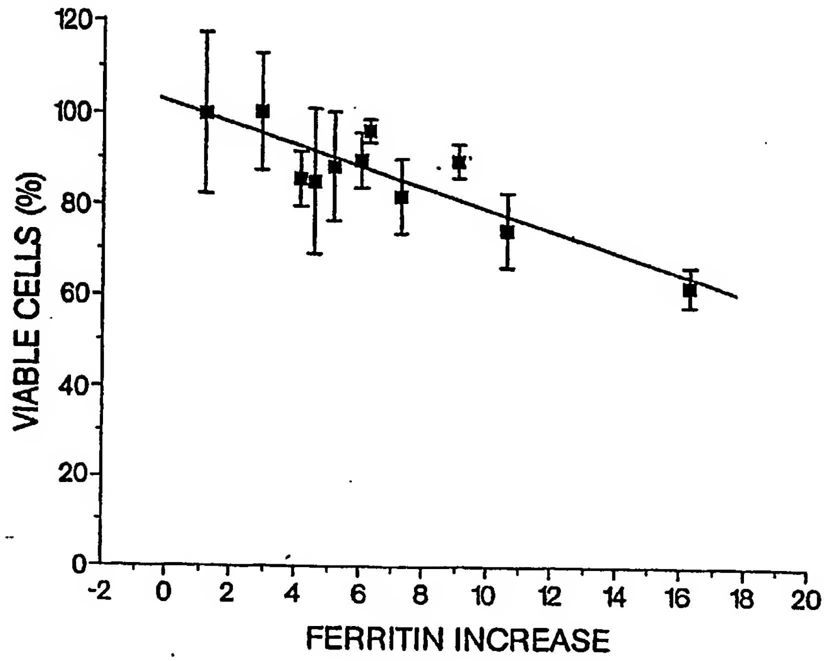
9
8
7
6
5
4
3
2
1
0
1/T2 (S-1)
0
2
4
6
8
10
12
14
16
18
20
FERRITIN INCREASE

Fig. 1b



120
100
80
60
40
20
0
-2
0
2
4
6
8
10
12
14
16
18
20
VIABLE CELLS (%)
FERRITIN INCREASE

Fig. 2

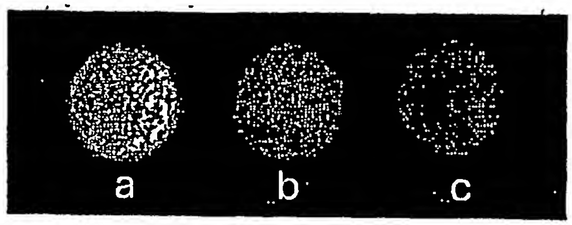


Fig. 3

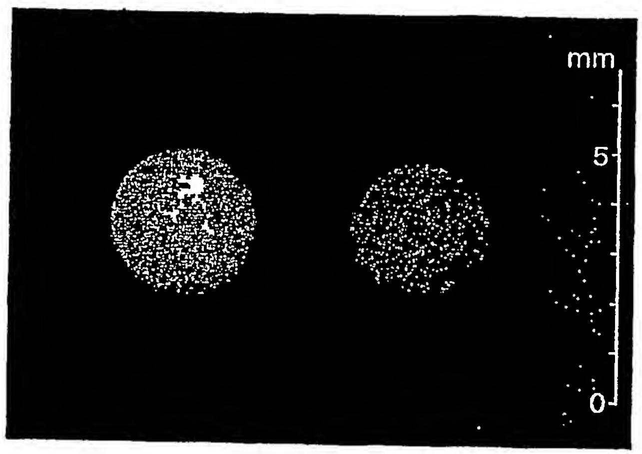


Fig. 4

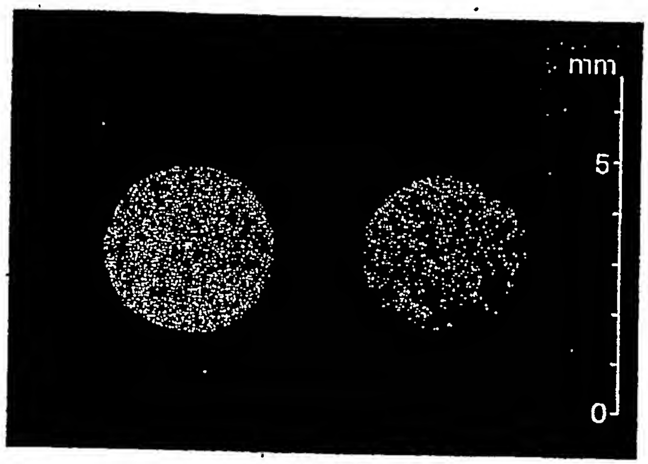


Fig. 5

Figure 6: Human Ferritin Heavy Chain Nucleic Acid Sequence (Acc. No. BC016009) (SEQ ID NO:1)

```
  1 ggggagacgt tcttcgccga gagtcgtcgg ggtttcctgc ttcaacagtg cttggacgga
 61 acccggcgct cgttccccac cccggccggc cgcccatagc cagccctccg tcacctcttc
121 accgcaccct cggactgccc caaggccccc gccgccgctc cagcgccgcg cagccaccgc
181 cgccgccgcc gcctctcctt agtcgccgcc atgacgaccg cgtccacctc gcaggtgcgc
241 cagaactacc accaggactc agaggccgcc atcaaccgcc agatcaacct ggagctctac
301 gcctcctacg tttacctgtc catgtcttac tactttgacc gcgatgatgt ggctttgaag
361 aactttgcca aatactttct tcaccaatct catgaggaga gggaacatgc tgagaaactg
421 atgaagctgc agaaccaacg aggtggccga atcttccttc aggatatcaa gaaaccagac
481 tgtgatgact gggagagcgg gctgaatgca atggagtgtg cattacattt ggaaaaaaat
541 gtgaatcagt cactactgga actgcacaaa ctggccactg acaaaaatga cccccatttg
601 tgtgacttca ttgagacaca ttacctgaat gagcaggtga aagccatcaa agaattgggt
661 gaccacgtga ccaacttgcg caagatggga gcgcccgaat ctggcttggc ggaatatctc
721 tttgacaagc acaccctggg agacagtgat aatgaaagct aagcctcggg ctaatttccc
781 catagccgtg gggtgacttc cctggtcacc aaggcagtgc atgcatgttg ggtttcctt
841 tacctttttct ataagttgta ccaaaacatc cacttaagtt ctttgatttg taccattcct
901 tcaaataaag aaatttggta ccctcaaaaa aaaaaaaaaa aaaaaaaaaa aaaaa
```

Figure 7: Human Ferritin Heavy Chain Amino Acid Sequence (Acc. No. AAH16009) (SEQ ID NO:2)

```
1   mttastsqvr qnyhqdseaa inrqinlely asyvylsmsy yfdrddvalk nfakyflhqs
61  heerehaekl mklqnqrggr iflqdikkpd cddwesglna mecalhlekn vnqsllelhk
121 latdkndphl cdfiethyln eqvkaikelg dhvtnlrkmg apesglaeyl fdkhtlgdsd
181 nes
```

Figure 8: Human Ferritin Light Chain cDNA Sequence (Acc. No. XM_050469) (SEQ ID NO:3)

```
  1 tgcatcaaaa agctttattt ccatttggtc caaggcttgt taggatagtt aagaaagctg
 61 cctattggct ggagggagag gcttaggcag aagccctatt actttgcaag ggcccttca
121 gaagtcgctg ggctcagaag gctcttagtc gtgcttgaga gtgagccttt cgaagagata
181 ctcgcccagc ccagcctccg ggccacccag cctgtggagg ttggtcaggt ggtcacccat
241 cttcttgata agcttcactt cctcatctag gaagtgagtc tccaggaagt cacagagatg
301 ggggtccgtg cgggcagaac ccagggcatg aagatccaaa agggcctggt tcagcttttt
361 ctccagggcc atggcagctt tcatggcgtc tggggtttta ccccactcat cttcagctgg
421 cttcttgatg tcctggaaga gagcgcggcc gccacgctgg ttttgcatct tcaggagacg
481 ctcgtagccc tcgcgcttct cctcggccaa ttcgcggaag aagtggctca cgccttccag
541 agccacatca tcgcggtcga aatagaagcc cagagagagg taggtgtagg aggcctgcag
601 gtacaaattg accaggctgt tgacggctgc ctccacgtcg gtggaataat tctgacgaat
661 ctgggagctc atggttggtt ggcaagaagg agctaaccac aaaaacggtg ctggcaggtc
721 ccagaagcag gagatggccg agaagatggt cccggaggtt gcaagcggag aggaaatcgg
781 agggcggtcg gaggctggaa gagagtcccc ggatctgttc cgtccaaaca ctgttgaagc
841 aagagacaga cccgcgggac
```

Figure 9: Human Ferritin Light Chain Amino Acid Sequence (Acc. No. XP_050469) (SEQ ID NO:4)

```
  1 mgvragrtqg mkiqkglvql flqghgsfhg vwgftplifs wlldvleesa aatlvlhlqe
 61 tlvalalllg qfaeevahaf qshiiaveie aqrevgvggl qvqidqavdg clhvggiilt
121 nlgahgwlar rs
```

Figure 10: Mus musculus ferritin heavy chain cDNA sequence (Acc. No. NM_010239.1) (SEQ ID NO:5)

```
  1 cagacgttct cgcccagagt cgccgcggtt tcctgcttca acagtgcttg aacggaaccc
 61 ggtgctcgac ccctccgacc cccgccggcc gcttcgagcc tgagcccttt gcaacttcgt
121 cgttccgccg ctccagcgtc gccaccgcgc ctcgccccgc cgccaccatg accaccgcgt
181 ctccctcgca agtgcgccag aactaccacc aggacgcgga ggctgccatc aaccgccaga
241 tcaacctgga gttgtatgcc tcctacgtct atctgtctat gtcttgttat tttgaccgag
301 atgatgtggc tctgaagaac tttgccaaat actttctcca ccaatctcat gaggagaggg
361 agcatgccga gaaactgatg aagctgcaga accagcgagg tggccgaatc ttcctgcagg
421 atataaagaa accagaccgt gatgactggg agagcgggct gaatgcaatg gagtgtgcac
481 tgcacttgga aaagagtgtg aatcagtcac tactggaact gcacaaactg gctactgaca
541 agaatgatcc ccacttatgt gacttcattg agacgtatta tctgagtgaa caggtgaaat
601 ccattaaaga actgggtgac cacgtgacca acttacgcaa gatgggtgcc cctgaagctg
661 gcatggcaga atatctcttt gacaagcaca ccctgggaca cggtgatgag agctaagctg
721 acttccccaa agccacgtga ctttactggt cactgaggca gtgcatgcat gtcaggctgc
781 cttcatcttt tctataagtt gcaccaaaac atctgcttaa gttctttaat ttgtaccatt
841 tcttcaaata aagaattttg gtaccc
```

Figure 11: Mus musculus ferritin heavy chain amino acid sequence (NP_034369.1) (SEQ ID NO:6)

```
  1 mttaspsqvr qnyhqdaeaa inrqinlely asyvylsmsc yfdrddvalk nfakyflhqs
 61 heerehaekl mklqnqrggr iflqdikkpd rddwesglna mecalhleks vnqsllelhk
121 latdkndphl cdfietyyls eqvksikelg dhvtnlrkmg apeagmaeyl fdkhtlghgd
181 es
```

Figure 12: Mus musculus ferritin light chain 1 cDNA sequence (NM_010240.1) (SEQ ID NO:7)

```
  1 cagcgccttg gaggtcccgt ggatctgtgt acttgcttca acagtgtttg aacggaacag
 61 acccggggat tcccactgta ctcgcttcca gccgccttta caagtctctc cagtcgcagc
121 ctccgggacc atctcctcgc tgccttcagc tcctaggacc agtctgcacc gtctcttcgc
181 ggttagctcc tactccggat cagccatgac ctctcagatt cgtcagaatt attccaccga
241 ggtggaagct gccgtgaacc gcctggtcaa cttgcacctg cgggcctcct acacctacct
301 ctctctgggc ttcttttttg atcgggatga cgtggctctg gaaggcgtag gccacttctt
361 ccgcgaattg gccgaggaga agcgcgaggg cgcggagcgt ctcctcgagt ttcagaacga
421 tcgcgggggc cgtgcactct tccaggatgt gcagaagcca tctcaagatg aatggggtaa
481 aacccaggag gccatggaag ctgccttggc catggagaag aacctgaatc aggccctctt
541 ggatctgcat gccctgggtt ctgcccgcac ggaccctcat ctctgtgact tcctggaaag
601 ccactatctg gataaggagg tgaaactcat caagaagatg ggcaaccatc tgaccaacct
661 ccgcagggtg gcggggccac aaccagcgca gactggcgcg ccccaggggt ctctgggcga
721 gtatctcttt gagcgcctca ctctcaagca cgactaggag gcctctgtac cttccaaggg
781 gctcccccct ctgctctgca ccagcccgcc ctgggacctc cacctgaatg aacctctcaa
841 gccactaggc agctttgtaa ccgtcctcca gcctctgtca agtcttggac caagtaaaaa
901 taaagctttt tgagaccccg
```

Figure 13: Mus musculus ferritin light chain 1 amino acid sequence (NP_034370.1) (SEQ ID NO:8)

```
  1 mtsqirqnys teveaavnrl vnlhlrasyt ylslgfffdr ddvalegvgh ffrelaeekr
 61 egaerllefq ndrggralfq dvqkpsqdew gktqeameaa lameknlnqa lldlhalgsa
121 rtdphlcdfl eshyldkevk likkmgnhlt nlrrvagpqp aqtgapqgsl geylferltl
181 khd
```

Figure 14: Mus musculus ferritin light chain 2 cDNA sequence (NM_008049.1) (SEQ ID NO:9)

```
  1 ggaagactgt aaaagtcttg tcattttgtt cagtgaagtc ccctcattca catcaccaag
 61 gatgatgaca gtctctccag tcgccgcagc ctccgggacc atctccttgc cgccttccgg
121 tcctaggacc agccagcccc gtcttcgcgg ttagctccat actccggatc agccatgacc
181 tctcagattc gtcagaatta ttccaccgaa gtggaagctg ccgtgaaccg cctggtcaac
241 ttgcacctgc gggcctctta cacctacctc tctctgggct tcttttttga tcgggatgac
301 gtggctttgg aaggcgtagg ccacttcttc cgcgaattgg ccgaggagaa gcgcgagggc
361 gcggagcgtc tcctcaagtt gcagaacgaa cgcgggggcc gtgcactctt ccaggatgtg
421 cagaagccat ctcaagatga gtggggtaaa accctggagg ccatccaagc tgccttgcgc
481 ctggagaaga acctgaacca ggccctcttg gatctgcacg ccctgggctc tgcccgcaca
541 gaccctcacc tctgtgactt cttggaaagc cacttcctgg ataaggaggt gaagctcatc
601 aagaagatgg gcaaccacct gaccaacctc cgtagggtgg caggggccaca accagtgcag
661 actggcgtgg cccaggcatc tctgggcgag tatctctttg agcgcctcac tctgaagcac
721 gactaggcct ctgtgccttc caaggggctc cctcctctgc tctgcaccga ccgcctcagc
781 acctccaccc gaatgaacct ctaaagccac taggcagctt tgtaaccgcc ctggagcctc
841 tcccaagtct tggaccaagt aaaaataaa
```

Figure 15: Mus musculus ferritin light chain 2 amino acid sequence (NP_032075.1) (SEQ ID NO:10)

```
  1 mtsqirqnys teveaavnrl vnlhlrasyt ylslgfffdr ddvalegvgh ffrelaeekr
 61 egaerllklq nerggralfq dvqkpsqdew gktleaiqaa lrleknlnqa lldlhalgsa
121 rtdphlcdfl eshfldkevk likkmgnhlt nlrrvagpqp vqtgvaqasl geylferltl
181 khd
```

Figure 16: Rattus norvegicus ferritin subunit H cDNA sequence (NM_012848.1) (SEQ ID NO:11)

```
  1 cgacagtgct tgaacggaac ccggtgctcg acccctccga cccccgccgg ccgctttgag
 61 cctgagccct ttgcaacttc gtcgctccgc cgctccagcg tcgcctccgc gcctcgccca
121 gccgccatca tgaccaccgc gtctccctcg caagtgcgcc agaactacca ccaggactcg
181 gaggctgcca tcaaccgcca gatcaacctg gagttgtatg cctcctacgt ctatctgtcc
241 atgtcttgtt attttgaccg ggatgatgtg gccctgaaga actttgccaa atactttctc
301 catcaatctc atgaagagag ggaacatgct gagaaactga tgaagctgca gaaccagcga
361 ggtggacgaa tcttcctgca ggatataaag aaacctgacc gtgatgactg ggagagcggg
421 ctgaatgcaa tggagtgtgc actgcacttg gaaaagagtg tgaatcagtc actactggaa
481 cttcacaaac tggctactga caagaatgat ccccacttat gtgacttcat tgagacgcat
541 tacctgaatg agcaggtgaa atccattaaa gaactgggtg accacgtgac caacttacgc
601 aagatgggag cccctgaatc tggcatggca gaatatctct ttgacaagca caccctggga
661 cacggtgatg agagctaagc tgacgtcccc aaggccatgt gactttactg gtcactgagg
721 cagtgcatgc atgtcaggct gcctttatct tttctataag ttgcaccaaa acatctgctt
781 aaaagttctt taatttgtac catttcttca aataaagaat tttggtaccc
```

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Figure 17: Rattus norvegicus ferritin subunit H amino acid sequence (NP_036980.1) (SEQ ID NO:12)

```
  1 mttaspsqvr qnyhqdseaa inrqinlely asyvylsmsc yfdrddvalk nfakyflhqs
 61 heerehaekl mklqnqrggr iflqdikkpd rddwesglna mecalhleks vnqsllelhk
121 latdkndphl cdfiethyln eqvksikelg dhvtnlrkmg apesgmaeyl fdkhtlghgd
181 es
```

Figure 18: Rattus norvegicus ferritin light chain 1 cDNA sequence (NM_022500.1) (SEQ ID NO:13)

NP_071945.1

```
  1 atgacctctc agattcgtca gaattattcc accgaagtgg aagctgccgt gaaccgcctg
 61 gtcaacttgc acctgcgggc ctcttacacc tacctctctc tgggcttctt ttttgatcgg
121 gatgacgtgg ctttggaagg cgtaggccac ttcttccgcg aattggccga ggagaagcgc
181 gagggcgccg agcgtctcct caagttgcag aacgaacgcg gggccgtgc actcttccag
241 gatgtgcaga agccatctca agatgagtgg ggtaaaaccc tggaggccat ggaagctgcc
301 ttggccctgg agaagaacct gaaccaggcc ctcttggatc tgcacgccct gggctctgcc
361 cgcacagacc ctcacctctg tgacttcttg gaaagccact tcctggataa ggaggtgaag
421 ctcatcaaga agatgggcaa ccacctgacc aacctccgta gggtgcaggg cccacaacca
481 gcgcagactg gcgtggccca ggcatctctg ggcgagtatc tctttgagcg cctcactctg
541 aagcacgact ag
```

Figure 19: Rattus norvegicus ferritin light chain 1 amino acid sequence (NP_071945.1) (SEQ ID NO:14)

```
  1 mtsqirqnys teveaavnrl vnlhlrasyt ylslgfffdr ddvalegvgh ffrelaeekr
 61 egaerllklq nerggralfq dvqkpsqdew gktleameaa lalekn1nqa lldlhalgsa
121 rtdphlcdfl eshfldkevk likkmgnhlt nlrrvqgpqp aqtgvaqasl geylferltl
181 khd
```

Figure 20: Homo sapiens transferrin receptor cDNA sequence (NM_003234) (SEQ ID NO:15)

```
   1 ggcggctcgg gacggaggac gcgctagtgt gagtgcgggc ttctagaact acaccgaccc
  61 tcgtgtcctc ccttcatcct gcggggctgg ctggagcggc cgctccggtg ctgtccagca
 121 gccataggga gccgcacggg gagcgggaaa gcggtcgcgg ccccaggcgg ggcggccggg
 181 atggagcggg gccgcgagcc tgtggggaag gggctgtggc ggcgcctcga gcggctgcag
 241 gttcttctgt gtggcagttc agaatgatgg atcaagctag atcagcattc tctaacttgt
 301 ttggtggaga accattgtca tatacccggt tcagcctggc tcggcaagta gatggcgata
 361 acagtcatgt ggagatgaaa cttgctgtag atgaagaaga aaatgctgac aataacacaa
 421 aggccaatgt cacaaaacca aaaaggtgta gtggaagtat ctgctatggg actattgctg
 481 tgatcgtctt tttcttgatt ggatttatga ttggctactt gggctattgt aaaggggtag
 541 aaccaaaaac tgagtgtgag agactggcag gaaccgagtc tccagtgagg gaggagccag
 601 gagaggactt ccctgcagca cgtcgcttat attgggatga cctgaagaga aagttgtcgg
 661 agaaactgga cagcacagac ttcaccagca ccatcaagct gctgaatgaa aattcatatg
 721 tccctcgtga ggctggatct caaaaagatg aaaatcttgc gttgtatgtt gaaaatcaat
 781 ttcgtgaatt taaactcagc aaagtctggc gtgatcaaca ttttgttaag attcaggtca
 841 aagacagcgc tcaaaactcg gtgatcatag ttgataagaa cggtagactt gtttacctgg
 901 tggagaatcc tgggggttat gtggcgtata gtaaggctgc aacagttact ggtaaactgg
 961 tccatgctaa ttttggtact aaaaaagatt ttgaggattt atacactcct gtgaatggat
1021 ctatagtgat tgtcagagca gggaaaatca cctttgcaga aaaggttgca aatgctgaaa
1081 gcttaaatgc aattggtgtg ttgatataca tggaccagac taaatttccc attgttaacg
1141 cagaactttc attctttgga catgctcatc tggggacagg tgacccttac acacctggat
1201 tcccttcctt caatcacact cagtttccac catctcggtc atcaggattg cctaatatac
1261 ctgtccagac aatctccaga gctgctgcag aaaagctgtt tgggaatatg gaaggagact
1321 gtccctctga ctggaaaaca gactctacat gtaggatggt aacctcagaa agcaagaatg
1381 tgaagctcac tgtgagcaat gtgctgaaag agataaaaat tcttaacatc tttggagtta
1441 ttaaaggctt tgtagaacca gatcactatg ttgtagttgg ggcccagaga gatgcatggg
1501 gccctggagc tgcaaaatcc ggtgtaggca cagctctcct attgaaactt gcccagatgt
1561 tctcagatat ggtcttaaaa gatgggtttc agcccagcag aagcattatc tttgccagtt
1621 ggagtgctgg agactttgga tcggttggtg ccactgaatg gctagaggga tacctttcgt
1681 ccctgcattt aaaggctttc acttatatta atctggataa agcggttctt ggtaccagca
1741 acttcaaggt ttctgccagc ccactgttgt atacgcttat tgagaaaaca atgcaaaatg
1801 tgaagcatcc ggttactggg caatttctat atcaggacag caactgggcc agcaaagttg
1861 agaaactcac tttagacaat gctgctttcc ctttccttgc atattctgga atcccagcag
1921 tttctttctg ttttgcgag  gacacagatt atccttattt gggtaccacc atggacacct
1981 ataaggaact gattgagagg attcctgagt tgaacaaagt ggcacgagca gctgcagagg
2041 tcgctggtca gttcgtgatt aaactaaccc atgatgttga attgaacctg gactatgaga
2101 ggtacaacag ccaactgctt tcatttgtga gggatctgaa ccaatacaga gcagacataa
2161 aggaaatggg cctgagttta cagtggctgt attctgctcg tggagacttc ttccgtgcta
2221 cttccagact aacaacagat ttcgggaatg ctgagaaaac agacagattt gtcatgaaga
2281 aactcaatga tcgtgtcatg agagtggagt atcacttcct ctctccctac gtatctccaa
2341 aagagtctcc tttccgacat gtcttctggg gctccggctc tcacacgctg ccagctttac
2401 tggagaactt gaaactgcgt aaacaaaata acggtgcttt taatgaaacg ctgttcagaa
2461 accagttggc tctagctact tggactattc agggagctgc aaatgccctc tctggtgacg
2521 tttgggacat tgacaatgag ttttaaatgt gatacccata gcttccatga gaacagcagg
2581 gtagtctggt ttctagactt gtgctgatcg tgctaaattt tcagtagggc tacaaaacct
2641 gatgttaaaa ttccatccca tcatcttggt actactagat gtctttaggc agcagctttt
2701 aatacagggt agataacctg tacttcaagt taaagtgaat aaccacttaa aaaatgtcca
2761 tgatggaata ttcccctatc tctagaattt taagtgcttt gtaatgggaa ctgcctcttt
2821 cctgttgttg ttaatgaaaa tgtcagaaac cagttatgtg aatgatctct ctgaatccta
2881 agggctggtc tctgctgaag gttgtaagtg gttcgcttac tttgagtgat cctccaactt
2941 catttgatgc taaataggag ataccaggtt gaaagacctc tccaaatgag atctaagcct
3001 ttccataagg aatgtagcag gtttcctcat tcctgaaaga aacagttaac tttcagaaga
3061 gatgggcttg ttttcttgcc aatgaggtct gaaatggagg tccttctgct ggataaaatg
3121 aggttcaact gttgattgca ggaataaggc cttaatatgt taacctcagt gtcatttatg
```

```
3181 aaaagagggg accagaagcc aaagacttag tatattttct tttcctctgt cccttccccc
3241 ataagcctcc atttagttct ttgttatttt tgtttcttcc aaagcacatt gaaagagaac
3301 cagtttcagg tgtttagttg cagactcagt ttgtcagact ttaaagaata atatgctgcc
3361 aaattttggc caaagtgtta atcttagggg agagcttttct gtccttttgg cactgagata
3421 tttattgttt atttatcagt gacagagttc actataaatg gtgttttttt aatagaatat
3481 aattatcgga agcagtgcct tccataatta tgacagttat actgtcggtt ttttttaaat
3541 aaaagcagca tctgctaata aaacccaaca gatactggaa gttttgcatt tatggtcaac
3601 acttaagggt tttagaaaac agccgtcagc caaatgtaat tgaataaagt tgaagctaag
3661 atttagagat gaattaaatt taattagggg ttgctaagaa gcgagcactg accagataag
3721 aatgctggtt ttcctaaatg cagtgaattg tgaccaagtt ataaatcaat gtcacttaaa
3781 ggctgtggta gtactcctgc aaaattttat agctcagttt atccaaggtg taactctaat
3841 tcccatttgc aaaatttcca gtacctttgt cacaatccta acacattatc gggagcagtg
3901 tcttccataa tgtataaaga acaaggtagt ttttacctac cacagtgtct gtatcggaga
3961 cagtgatctc catatgttac actaagggtg taagtaatta tcgggaacag tgtttcccat
4021 aattttcttc atgcaatgac atcttcaaag cttgaagatc gttagtatct aacatgtatc
4081 ccaactccta taattcccta tcttttagtt ttagttgcag aaacattttg tggtcattaa
4141 gcattgggtg ggtaaattca accactgtaa aatgaaatta ctacaaaatt tgaaatttag
4201 cttgggtttt tgttaccttt atggtttctc caggtcctct acttaatgag atagcagcat
4261 acatttataa tgtttgctat tgacaagtca ttttaattta tcacattatt tgcatgttac
4321 ctcctataaa cttagtgcgg acaagtttta atccagaatt gacctttga cttaaagcag
4381 agggactttg tatagaaggt ttgggggctg tggggaagga gagtcccctg aaggtctgac
4441 acgtctgcct acccattcgt ggtgatcaat taaatgtagg tatgaataag ttcgaagctc
4501 cgtgagtgaa ccatcatata aacgtgtagt acagctgttt gtcatagggc agttggaaac
4561 ggcctcctag ggaaaagttc atagggtctc ttcaggttct tagtgtcact tacctagatt
4621 tacagcctca cttgaatgtg tcactactca cagtctcttt aatcttcagt tttatcttta
4681 atctcctctt ttatcttgga ctgacattta gcgtagctaa gtgaaaaggt catagctgag
4741 attcctggtt cgggtgttac gcacacgtac ttaaatgaaa gcatgtggca tgttcatcgt
4801 ataacacaat atgaatacag ggcatgcatt ttgcagcagt gagtctcttc agaaaaccct
4861 tttctacagt tagggttgag ttacttccta tcaagccagt acgtgctaac aggctcaata
4921 ttcctgaatg aaatatcaga ctagtgacaa gctcctggtc ttgagatgtc ttctcgttaa
4981 ggagtagggc cttttggagg taaaggtata
```

Figure 20 con't